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The crustacean cuticle: structure, composition and mineralization

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TABLE OF CONTENTS

1. ABSTRACT

Crustaceans have a rigid exoskeleton, which is made of a layered cuticle, covering the soft body parts for protection from conspecific competitors and/or interspecific predators. Calcium carbonate adds rigidity to the crustacean cuticle, which consequently means that growth only occur at each molt. The current study presents a review of existing literature on crustacean exoskeleton cuticle physiology and biochemistry in relation to the molting process with special reference to calcification. As a result, research matter where knowledge remains limited has been identified during the molting process, including 1) whether the same or different epithelial cells are responsible for the decomposition and/or reconstruction of chitin and proteins, 2) how calcium carbonate levels are regulated at the cellular level during transfer between the cuticle and body organs, and 3) what factors maintain the amorphous state of calcium carbonate following deposition in the exoskeleton and temporary storage organs. The identification of these areas of focus provides a basis on which targeted future research may be developed, and potentially applied to other invertebrate or even vertebrate processes.

2. INTRODUCTION

Crustaceans belong to the phylum of Arthropods, which have the largest number of species, including insects, spiders and so on. Arthropods have a cuticle system at the outermost boundary of the body (i.e. exoskeleton), separating it from the external environment (1). The cuticle has different functions based on species-specific morphology, including firstly defense from other animals and infectious diseases, and locomotion, and secondly reproduction, and/or the reception of external stimuli, such as chemicals and temperature, which are assisted by epithelial cells and muscles. In different species, such functions are achieved through the specialization of organs to have characteristic morphology and specific apparatus. However, in Arthropods calcification of the cuticle seems to occur only in crustaceans. While a calcified cuticle adds extra weight to the body, this does not seem to affect the behavior of aquatic crustaceans significantly. In contrast, most terrestrial Arthropods, especially insects, might have altered not to keep calcium carbonate during the evolution from aquatic to terrestrial environments, considering that insects have evolved from crustaceans, which has recently been clarified by the studies on molecular phylogeny (2, 3),

although this estimation is possible on the assumption that crustaceans obtained the ability of calcification before insects diverged from crustaceans. However, most terrestrial crustaceans still retain calcium carbonate in the cuticle (4). Here, the characteristics of the crustacean cuticle are described, including structure, composition and mineralization based on existing literature, in order to highlight aspects of processes that still require detailed investigation.

3. PHYSICAL STRUCTURE OF THE CUTICLE

Crustaceans belong to the phylum of Arthropods, which are invertebrates meaning that they do not have a backbone. In general, most crustaceans inhabit aqueous environments, with some living on land. Crustaceans usually have a hard exoskeleton to maintain species-specific morphology of the body, which protects their soft body parts from enemies and various kinds of diseases. The physical structure of the crustacean exoskeleton is characteristic in that it is made of a cuticle. The cuticle consists of four layers: the epicuticle, exocuticle, endocuticle, and a membranous layer from the outside to the inside (5-7). The outermost epicuticle layer is thin (1-5 micrometers), usually unmineralized and waterproofed by lipids, serving as a barrier to the outside world. On the other hand, the exocuticle is usually calcified, with an uneven distribution of minerals, constituting about one-fifth of the entire integument (8-10), as for example for the crab, *Cancer pagurus*. In the exocuticle, there are three different sites for mineral deposition: within pore canals, between chitin-protein fibrils, and within fibrils. The epicuticle and exocuticle are formed before molting (or ecdysis), while the mineralization of the exocuticle occurs after ecdysis (7). The endocuticle forms after ecdysis, and comprises the main and thickest calcified layer of the whole cuticle (7). The endocuticle consists of numerous layered lamellae. As in other Arthropods, the chitin-protein complex fibers of the lamellae are aligned in a plane parallel to the surface of exoskeleton (6). In successive planes, the fiber orientation is rotated at a constant angle to form a helicoidal architecture, in which a 180-degree rotation of fiber direction constitutes a single lamella (6).

Recent microscopic studies on the structure of cuticle in American lobster, *Homarus americanus*, revealed that there is another type of chitin-protein fibers perpendicular to the cuticle surface with honeycomblike structure originating from the pore-canal system (11-15). These characteristic structures strengthen the cuticle mechanically. In the American lobster, a small amount of crystalline calcite exists only at the outermost thin layer region in the exocuticle and is associated with the chitin-protein fibers oriented perpendicular to the surface. The c-axis of the calcite crystals is also oriented perpendicular to the surface. The mode of calcification in the cuticle was investigated with the blue crab, *Callinectes sapidus*, using scanning electron microscope (SEM) and transmission electron microscope (TEM). Calcification starts at the epicuticle/exocuticle boundary and at the distal and proximal margins of the interprismatic septa 3 hours after molting, and then extends bidirectionally until the two fronts meat 5-8 hours after molting (16). Eventually, calcification is accomplished at the end of the postmolt period.

4. CHEMICAL COMPOSITION OF THE CUTICLE

The cuticle consists of three major components: chitin, proteins and calcium carbonate together with various minor components including proteoglycans, lipids and other inorganic materials (5-7). The relative proportions of these three components vary depending on the species, and location on the body, even in a single individual (17). In general, harder cuticles have higher calcium carbonate content. The cuticle contains various ionized forms of trace elements that originate from the epithelial cells, such as magnesium (Mg), phosphorus (P), and sulfur (S). However the chemical forms of P and S remain unclear, with the majority existing as phosphates and sulfates, respectively, while a small portion may also be involved in organic components as esters.

Chitin is a polysaccharide forming a fibrous architecture, which becomes a scaffold for calcium carbonate deposition (5-7). Chitin is synthesized by chitin synthase at the apical side of epithelial cell membranes. Proteins are also synthesized by epithelial cells, and are subsequently secreted to the cuticle. A number of different proteins have been identified, most of which have an ability to bind to chitin to form a chitin/protein complex (17) and will be described in detail in the following sections.

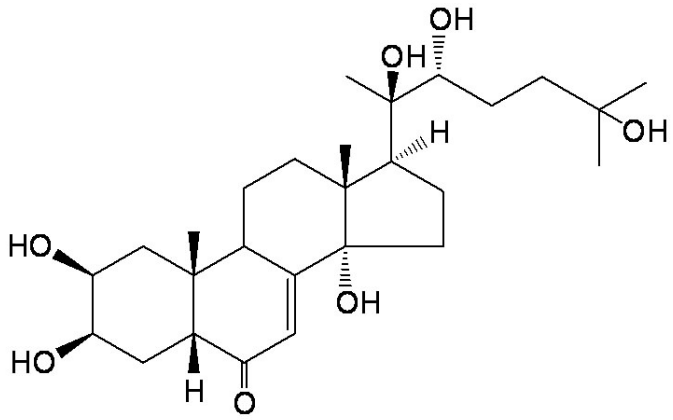
5. DECOMPOSITION AND FORMATION OF THE CUTICLE DURING MOLTING

5.1. Endocrine regulation of molting

The exoskeleton of insects and crustaceans inhibits growth, hence molting is essential to allow growth (7). The exoskeleton of crustaceans is a hard shell made of a cuticle, within which all organs are located in their respective positions, including the nervous system, digestive tract, respiratory organs, and hepatopancreas. The exoskeleton of crustaceans contains a large amount of the inorganic compound calcium carbonate, which reduces flexibility and elasticity. As a result, crustaceans cannot grow without molting. On the other hand, while insects have a cuticle structure similar to crustaceans, they do not have this inorganic component, resulting in their being more flexible. As a consequence, insects are able to grow a certain extent during the larval instar stages, whereas crustaceans grow stepwise via molting (1).

The hormone responsible for molting was first isolated from the silkworm, *Bombyx mori*, after much experimental effort across a long period of time (18), and later its chemical structure was determined by X-ray crystallography (19). The crustacean molting hormone, named crustecdysone, was subsequently isolated from a spiny lobster, *Jasus lalandei*, and identified as the same compound to the insect molting hormone, 20-hydroxyecdysone (20) (Figure 1a). Thus, the molting hormone was found to be common between insects and crustaceans. The organ that produces the molting hormone

a)



b)

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                1         10        20        30        40
Cam-MIH         RVINDECPNLIGNRDLYKKVEWICEDCSNIFRKTGMASLC

                41        50        60        70
                RRNCFFNEDFVWCVHATERSEELRDLEEWVGILGAGRD(OH)
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Figure 1. Hormones that control molting in crustaceans. a) molting hormone (20-hydroxyecdysone), b) molt-inhibiting hormone. Cam means a crab, *Carcinas maenas*.

in crustaceans was identified as a pair of Y-organs, which corresponds to the prothoracic glands in insects. The Y-organ is located on the ventral side of the head/thorax region, and is a cluster of several hundred of cells that are specialized in producing the molting hormone (21).

Interestingly, the synthesis of the molting hormone in crustaceans is not controlled in the same manner as in insects. Over 100 years ago, Zeleny first demonstrated that the bilateral eyestalk ablation in crustaceans induced precocious molting (22), suggesting the existence of a factor that inhibits molting. Later, this factor, termed molt-inhibiting hormone (MIH), was extracted and purified to homogeneity from the sinus gland of the crab, *Carcinus maenas*, which is a neurohemal organ. The structure of the hormone was subsequently determined to be a 77-amino-acid-residue peptide (Figure 1b) (23). MIH is a member of the crustacean hyperglycemic hormone (CHH) family (24), which comprises CHH, MIH, vitellogenesis-inhibiting hormone and mandibular organ-inhibiting hormone. MIH has been characterized in over ten crustacean species (25). All MIH molecules are composed of about seventy amino acid residues and six cysteine residues, which form three intramolecular disulfide bonds. In contrast, the prothoracicotropic hormone (PTTH) of insects, a glycoprotein, is produced by a limited number of neurosecretory cells in the brain, and transferred through axons to the corpora allata for storage. Subsequently, PTTH is released into the hemolymph, stimulating the prothoracic glands to produce ecdysteroids. Thus, the Y-organs of crustaceans are under inhibitory regulation, while the prothoracic glands of insects are under stimulatory regulation.

5.2. Temporary storage of calcium carbonate during molting

Calcium carbonate in the cuticle helps maintain the hardness of the exoskeleton; however this hardness may interfere with the molting process. Crustaceans therefore reduce calcium carbonate content levels in the cuticle before molting to increase flexibility. While both the exocuticle and endocuticle are calcified, it is the calcium carbonate in the endocuticle that is mainly dissolved (Figure 2). Although it is unclear how calcium carbonate is dissolved in the cuticle, acid may play a role. It is also unclear how calcium and bicarbonate ions, derived from calcium carbonate, are transported from the cuticle to the hemolymph through the epithelium. If these ions are transported through epithelial cells, a special system to decrease calcium concentrations in the cell is required, because calcium concentrations are usually maintained at very low levels.

The site of the calcium storage depends on the crustacean species (26). For example, in crustaceans such as crayfish and lobster, most calcium and bicarbonate ions are transferred to the stomach, where calcium carbonate is re-deposited to form a pair of gastroliths (27). Deposition occurs at two symmetrical locations in the frontal part of the stomach, where columnar epithelial cells are clustered to form a round tissue, termed the gastrolith disk. The gastrolith disk is easily distinguished from other parts of the stomach tissue, even during the intermolt stage when

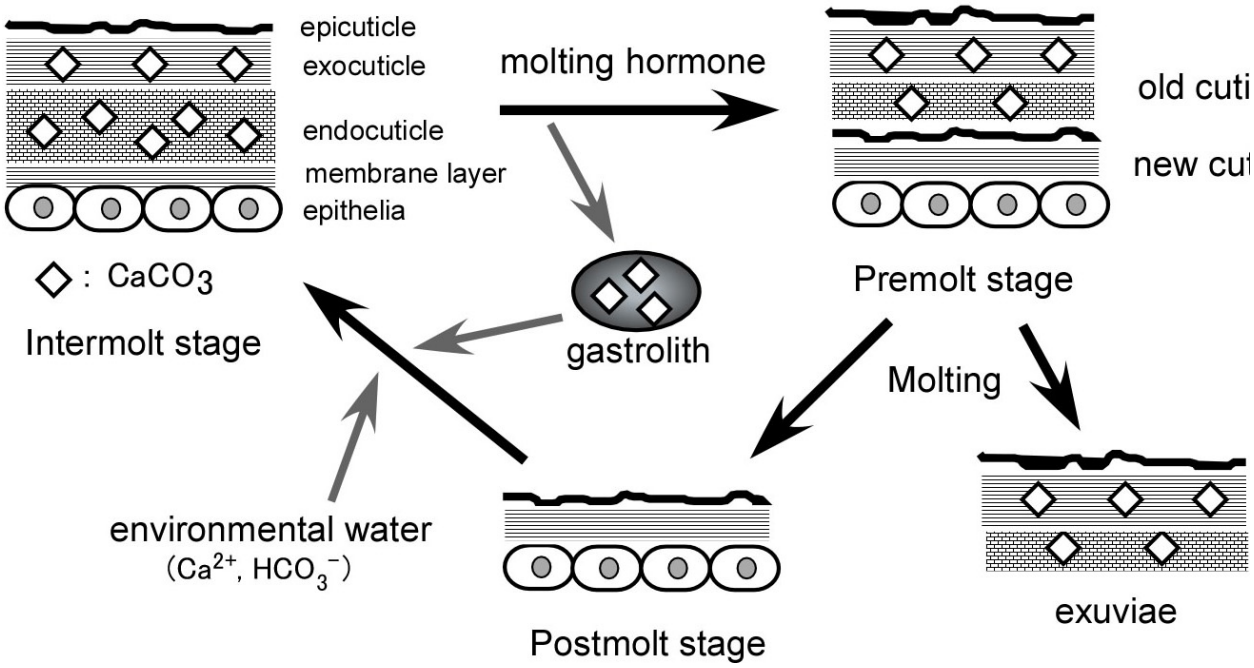

Figure 2. Calcium movement in the body before (premolt stage) and after molting (postmolt stage) in the crayfish.

gastroliths are not formed. The inner wall of the stomach is covered with cuticle, and gastroliths are formed between the single-layer epithelium and the cuticle. Calcium and bicarbonate ions may be transferred from the hemolymph to the cuticle, which is a similar process to that occurring at the level of the exoskeleton. Gastrolith formation occurs just after the initiation of the premolt stage, with rapid growth before ecdysis. After ecdysis, gastroliths are re-dissolved, and calcium and bicarbonate ions are transferred to the newly formed cuticle for deposition. Thus, gastroliths serve as temporary storage sites for calcium carbonate (28).

In some terrestrial crustaceans, such as *Orchestia cavimana*, calcium carbonate is deposited in storage organs of the midgut, termed ceca (29). In some terrestrial isopods, such as the woodlice *Porcellio scaber* and *Armadillidium vulgare*, calcium carbonate is deposited at a sternal site (30). Other crustaceans store calcium carbonate in the hepatopancreas or hemolymph (31). In all cases, calcium carbonate deposits serve as temporary storage sites similar to that of gastroliths.

5.3. Synthesis and decomposition of chitin

The main component of organic materials in the cuticle is chitin (3). Chitin is a beta-(1-4)-conjugated homopolymer of *N*-acetyl-D-glucosamine (GlcNAc) (32). There are two conformational polymorphs, alpha- and beta-chitin, with anti-parallel and parallel structures of carbohydrate chains, respectively (32). Alpha-Chitin is thermodynamically more stable than beta-chitin (32). It is speculated that chitin is synthesized by chitin synthase, which is located at the epithelial surface directly adjoining the cuticle, and is directly released into the extracellular space, although to date, chitin synthase has yet to be identified in crustaceans.

Chitin is decomposed partially during the premolt stage, to separate the old cuticle from the new cuticle, and for reuse of hydrolyzed *N*-acetylglucosamine, as in insects (33). The hydrolysis of chitin is performed by chitinase, of which 14 molecular types have been identified in crustaceans (34). Multiple chitinase genes are usually present in a single individual, and some of them are responsible for the decomposition of chitin in the cuticle, while others are related to the digestion of food (34).

6. REGULATION OF CALCIFICATION IN THE CUTICLE AND TEMPORARY STORAGE ORGANS

6.1. Acidic macromolecules

In general, acidic macromolecules are considered to play a crucial role in biomineralization (35). Table 1 shows some representative cuticle proteins and peptides thus far characterized by direst protein sequencing and/or cDNA cloning. Early research on the characterization of crustacean cuticle proteins and peptides identified various molecular species as proteins (36-38). Urea-extractable materials from the calcified exoskeleton of the lobster, *Homarus americanus*, contain various kinds of proteins as detected on a two-dimensional electrophoresis gel. Some of the proteins were sequenced, and it was found that five proteins with pI values of about 4 and molecular masses of about 12 kDa had very similar amino acid sequences to each other. On the other hand, different proteins were isolated from arthrodial part of cuticle of the same species and found to contain the Rebers-Riddiford (R-R) consensus sequence for chitin binding, which was first characterized in insect cuticle peptides (39, 40). The proteins derived from calcified exoskeleton may be involved in calcification of cuticle, although no direct proof was indicated. From the

Table 1. Cuticle proteins and peptides identified from crustaceans by protein sequencing or deduction from cDNA sequences

Protein or peptide	Species	No. of residues	Accession No.[2]	Reference
HA-AMP1B	*Homarus americanus*	105	P81385	37
AM1274	*Cancer pagurus*	116	P81579	38
CPAM1199	*Cancer pagurus*	108	P81577	38
CAP-1	*Procambarus clarkii*	78	AB103035	41
CAP-2	*Procambarus clarkii*	62	AB167814	43
Casp-2	*Procambarus clarkii*	118	AB292778	45
crustocalcin	*Marsupenaeus japonicus*	831	AB114444	46
AMP6.0	*Callinectes sapidus*	111[1]	AY752734	49
AMP8.1	*Callinectes sapidus*	134[1]	AY752733	49
CP8.2	*Callinectes sapidus*	107[1]	AY752736	49
CP8.5	*Callinectes sapidus*	105[1]	AY752735	49
CP14.1	*Callinectes sapidus*	144[1]	DQ288151	50
AMP13.4	*Callinectes sapidus*	135[1]	DQ288147	50
AMP16.5	*Callinectes sapidus*	163[1]	DQ288152	50

[1] including a signal peptide. [2] DDBJ/EMBL/GenBank accession number

crab, *Cancer pagurus*, twelve proteins from the calcified exoskeleton and five from arthrodial membranes were purified and sequenced (38). One of them from the former proteins was identical with one of the latter proteins. Several proteins are similar to the lobster cuticle proteins in amino acid sequence and they have either two or four copies of an 18-residue sequence motif (termed crust-18), XLXGPSGBBDGXXXQB, where X is any amino acid residue and B is a hydrophobic residue, which is a peculiar sequence in crustaceans. But, its function remains to be solved.

Two novel cuticle peptides associated with calcification, termed calcification-associated peptide (CAP)-1 and -2, were first purified and identified from the acid-insoluble fraction of the crayfish exoskeleton, *Procambarus clarkii* (41-43). Purification was performed using a simple calcification-inhibition assay, which was modified for small scales from the method developed previously (44). This assay actually evaluates the ability of a substance to bind to calcium carbonate by assessing the inhibitory activity of calcium carbonate precipitation from its supersaturated solution (36). CAP-1 and -2 are acidic peptides consisting of 78 and 62 amino acid residues, respectively, with CAP-1 being more active than CAP-2. The C-terminal part of CAP-1 is extremely rich in acidic amino acid residues, an Asp repeat and phosphorylated Ser at position 70, which were thought to be responsible for the high rates of calcification inhibitory activity (35). Both peptides share the R-R consensus sequence at the central part of each molecule. In fact, recombinant peptides of CAP-1 and -2 also indicated chitin-binding ability *in vitro*.

From the acid-soluble fraction of the crayfish exoskeleton, two calcification inhibitory proteins were isolated and termed calcification-associated soluble protein (Casp) -1 and -2 (45). Casp-2 was fully characterized using cDNA cloning, and found to have the R-R consensus sequence. Casp-2 is a 105-amino acid residue protein with pI 4.25, with fewer acidic amino acid residues than CAP-1 or -2. Recombinant Casp-2 also indicated chitin-binding ability *in vitro*; however the affinity to chitin was weaker than CAP-1 and -2, which may be consistent with the evidence that Casp-2 was extracted with an acidic solution.

Crustacean cuticle proteins have also been characterized at both protein and cDNA levels. For example, in the kuruma prawn, *Marsupenaeus japonicus*, three proteins were identified: DD4 (later renamed crustocalcin) (46), DD5 (47) and DD9 (48). Crustocalcin was deduced from the nucleotide sequence of cDNA, and consists of 831 amino acid residues, which include an R-R like domain, a Glu-rich domain and a Pro-rich domain, in this order from the N-terminus. The Glu-rich domain contains eight consecutive Glu residues, which is also found in human bone sialoprotein and is considered to be responsible for calcification of bone. It was found that the central part of the Pro-rich domain has an ability to bind Ca^{2+} ions (46). Immunohistochemical analysis showed the localization of this protein in the endocuticle, suggesting that crustocalcin is associated with the calcification of the endocuticle after ecdysis.

A new approach to identify cuticle proteins has recently been performed using an expressed sequence tag (EST) library derived from epithelia. In the example of the blue crab, *Callinectes sapidus*, four cuticle proteins were identified: AMP8.1 and AMP6.0 from arthrodial membranes, and CP8.2 and CP8.5 from calcified part by using the characteristics of having the R-R consensus sequence (49). Subsequently, eight differentially expressed genes encoding cuticle proteins were identified from the same species (50). More extended survey of cuticle proteins was performed using an EST library from *C. sapidus*, resulting in obtaining complete sequences of 25 transcripts, which were classified into three groups having the R-R consensus sequence, the crust-18 sequence, and the others. Phylogenic analyses of the proteins belonging to the former two groups were performed in comparison with similar molecules of insects or within crustacean species, and their spatial and temporary gene expressions were examined in detail, leading to their putative function in cuticle formation and calcification (51). From the crab, *Portunus pelagicus*, a large variety of genes associated with cuticle formation were identified and their differential expressions across the molting stages were clarified using a cDNA microarray chip (52). Some of the cuticle proteins share the sequence characteristics with those identified from *H. americanus* and *C. pagurus*. However, it is not clear whether these genes are associated with calcification or not.

The acidic property of organic matrices has been considered important for the interaction with calcium ions

and/or calcium carbonate (35). Therefore, acidic macromolecules are believed to be essential for calcification, although a detailed systematic study on the structure-function relationship is required. A structure-function relationship study was performed using CAP-1 (53), in which recombinant peptides were prepared: a dephosphorylated peptide of CAP-1, a peptide replaced by Asp at Ser70, a peptide truncated by the C-terminal 16 residues, a peptide truncated by the N-terminal 16 residues, and a peptide truncated by both N- and C-terminal 16 residues. These peptides were subjected to the previously described calcification inhibition assay. The results showed that phosphorylation contributes to enhancing inhibitory activity, while the substitution of Ser by Asp partially enhances activity, and the C-terminal acidic part is most important for the activity. Based on these results, the C-terminal partial peptide and its analogues were chemically synthesized and assessed for calcification inhibitory activity (54). The results showed that 1) calcification activity does not depend on the amino acid sequence, but on the number of acidic amino acid residues in a peptide (i.e. the more acidic amino acid residues that a peptide has, the higher the activity), 2) peptide conformation is not important for activity as far as an oligopeptide is concerned, and 3) a peptide containing Asp is more active than a peptide containing Glu when the total number of acidic amino acid residues are equal. It remains unclear whether these results are also applicable to proteins. However, considering the minimal sequence homology among matrix proteins from various biological sources, molecular evolution may have occurred to remain only acidic in nature. While this issue is very important in the research of biomineralization, more data are required to reach definite conclusions.

6.2. Organic matrix contributing to the formation of gastroliths

Gastroliths are formed in the stomach for the temporary storage of calcium carbonate of the exoskeleton during the premolt stage. A protein was identified from the EDTA-soluble fraction of gastroliths in the crayfish, *C. quadricarinatus*, which was named GAP 65 based on its molecular mass (65 kDa) on an SDS-PAGE gel (55). A cDNA encoding GAP 65 was cloned using partial amino acid sequences, and from the nucleotide sequence the entire amino acid sequence was deduced. GAP 65 has three domains: a chitin-binding domain, a low-density lipoprotein receptor class A domain, and a polysaccharide deacetylase domain. However, the association of the latter two domains with gastrolith formation remains unknown. In *in vitro* precipitation experiment, GAP 65 induces the formation of amorphous calcium carbonate (ACC). However, in an RNAi experiment, the repression of the production of GAP 65 did not result in the repression of the formation of gastroliths, nor did it alter the crystal polymorph. In fact, only a small change was induced in the entire morphology of gastroliths (55). The GAP 65 gene is specifically expressed at the gastrolith disk and subepidermal tissue throughout the entire molting stage. The transcript level is low at the postmolt and intermolt stages, but high at the premolt stage, further supporting the association of GAP 65 with gastrolith formation.

In a different species of the crayfish, *P. clarkii*, an acid-insoluble, but SDS-soluble, protein was isolated and named gsstrolith matrix protein (GAMP) (56, 57). The deduced amino acid sequence, which was obtained from the nucleotide sequence of a cDNA encoding GAMP that was cloned based on the partial amino acid sequences, indicated that it consists of 487 amino acid residues, including two atypical tandem-repeated sequences. One sequence is 17 repetitions of 10 amino acid residues, Q-V/A-A-Q-E-Q-A-Q-E-G, which is present at the N-terminal side. The other sequence is 15 repetitions of 5 amino acid residues, G-S-X-S/G-F, at the C-terminal side. Although the meaning of these repetitive sequences remains unclear, GAMP has some characteristics related to calcification (58). It has the capacity to bind to chitin, and has calcification inhibitory activity. The *GAMP* gene is expressed only in the gastrolith disk and only at the premolt stage. Furthermore, the expression of this gene in the gastrolith disk is greatly enhanced by the molting hormone, when cultured *in vitro*. Immunohistochemistry using an anti-GAMP antiserum indicated that GAMP is located uniformly in the gastrolith and epithelial cells, suggesting that GAMP is constantly produced, secreted and incorporated into the gastroliths (59). All these results strongly indicate that GAMP is deeply associated with gastrolith formation.

6.3. Organic matrix contributing to the calcification in ceca

In the terrestrial amphipod, *Orchestia cavimana*, the calcareous concretions in the posterior ceca were found to contain a 23-kDa protein on an SDS-PAGE gel and named Orchestin, which was considered to be a noteworthy component in the EDTA-soluble fraction (60). Based on the N-terminal sequence, a cDNA encoding Orchestin was cloned (61). From the deduced sequence, the molecular mass and pI of Orchestin were estimated to be 12.4 kDa and 4.4, respectively. It has been found that the acidity of this protein may reduce the mobility on the SDS-PAGE gel. When the *orchestin* gene was subject to *in situ* hybridization, spatiotemporal analysis demonstrated that it was expressed at the storage organ cells mainly during the premolt stage. Hence, the expression of this gene is induced by the molting hormone. Immunohistochemistry using an anti-Orchestin antiserum revealed the exclusive existence of Orchestin as an organic-matrix component (62). It was found that Orchestin is phosphorylated at Ser and Tyr residues, the former being necessary for calcium binding (63). The *orchestin* gene is also expressed at the postmolt stage, and therefore Orchestin may also be used for the reabsorption of calcium carbonate in ceca. These results strongly suggest that Orchestin is a key molecule in the calcium carbonate precipitation process.

6.4. Organic matrices responsible for amorphous calcium carbonate

Calcium carbonate in the cuticle may be either amorphous or crystalline (6). Amorphous calcium carbonate (ACC) and calcite may on occasion coexist in the cuticle of a single individual. This phenomenon has also been observed in animals other than Arthropods, for example calcareous sponges and ascidians (64). In general,

it is difficult to maintain an amorphous state because amorphous calcium carbonate has a tendency to transform to crystal form, such as calcite, aragonite or vaterite (65). In biominerals, there are two types of ACC (66); the first is a transient state that quickly transforms into crystals, while the second is maintained in a stable state for long periods. In crustaceans, ACC takes the form of the second state. Furthermore, previous research has demonstrated that phosphate may be important for maintaining the amorphous state (67). In any case, noticeably high phosphorus content levels have been found in the cuticle. Of note, precipitates for temporary storage, such as gastroliths and deposits in the ceca, are almost exclusively composed of ACC. In fact, ACC has the characteristic of being more easily dissolved than crystalline forms (68). Hence, crustaceans utilize ACC effectively, because during both premolt and postmolt periods the efficient transfer of calcium inside the body is required.

In crustaceans, molecules other than ions are suspected to play a role in the stabilization of the amorphous state. For example, in *in vitro* precipitation of calcium carbonate, ACC formation was induced by GAP 65, the previously described gastrolith protein from *C. quadricarinatus* (55). However, while inhibition of the GAP 65 gene by RNA interference caused morphological deformities, it failed to change ACC to crystalline form. These results suggest that the presence of additional unidentified factor(s) may be required to induce ACC in the gastrolith. It is speculated that highly phosphorylated proteins may serve as ACC stabilizers on the basis of the finding that the extract of crayfish exoskeleton including phosphorylated induced ACC formation *in vitro* (69). This speculation was supported by the similar ability of a single phosphoamino acid.

Another approach to solve this problem has been done by direct identification of phosphorus-containing compounds from the exoskeleton and gastroliths of the crayfish, *P. clarkii*. ^{31}P NMR, ^{1}H NMR and ^{13}NMR coupled with two-dimensional NMR analyses of the partially purified preparations clarified the presence of two phosphorus-containing compounds, phosphoenolpyruvate and 3-phosphoglycerate (70). These compounds could induce ACC *in vitro* at 1mM and maintained amorphous state for at least several days. Interestingly, both compounds are intermediates in the glycolytic pathway. Therefore, the epithelial cells responsible for calcified tissue formation have a specific ability to excrete these compounds actively and constantly by an unknown mechanism for ACC formation.

7. PERSPECTIVE

The formation and decomposition of the cuticle is a dynamic process occurring at every cycle of molting. This process has attracted broad scientific interest, resulting in targeted research of Arthropod growth and mineralization processes, particularly in relation to the strict endocrine control through the functioning of epithelial cells. However, questions still remain about many aspects of the molting process. For example, before molting, the molting hormone acts on epithelial cells to produce chitin and proteins to construct a new cuticle, while in parallel inducing the partial decomposition of the old cuticle. Research is required to clarify whether these two processes are performed by the same or different cells. Furthermore, the translocation of large amounts of calcium carbonate inside the body before and after molting is another interesting feature of the crustacean molting process. Epithelial cells facilitate the transfer of calcium carbonate from the cuticle to the hemolymph before molting and back again after molting. Since the cell usually maintains low calcium levels, a special mechanism to reduce calcium concentrations in each epithelial cell during translocation might be expected and further investigation will be required. With respect to the calcium transport system, calcium carbonate that is deposited in the cuticle and specific temporary storage organs is often amorphous, possibly because amorphous calcium carbonate is more easily dissolved than the crystalline form. It is therefore logical that to maintain the amorphous state other factors may be present, which have recently been identified as phosphorus-containing compounds (70). This review of the existing published research on the crustacean molting process has identified several areas in which information remains sparse, yet which may positively contribute to improving current knowledge of crustacean cuticle physiology and biochemistry.

8. ACKNOWLEDGEMENTS

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Abbreviations: ACC: amorphous calcium carbonate, CAP: calcification-associated peptide, Casp: calcification-associated soluble peptide, CHH: crustacean hyperglycemic hormone, GAMP: gastrolith matrix protein, GlcNAc: *N*-acetyl-D-glucosamine, MIH: molt-inhibiting hormone, PTTH: prothoracicotropic hormone, R-R: Rebers-Riddiford, SDS-PAGE: sodium dodecylsulfate-polyacrylamide gel electrophoresis

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