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Immunolocalization of Gastrolith Matrix Protein (GAMP) in the Gastroliths and Exoskeleton of Crayfish, *Procambarus clarkii*

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ABSTRACT—Gastrolith matrix protein (GAMP) is a novel protein purified from gastroliths of the crayfish, *Procambarus clarkii*, and has been suggested to be associated with calcium carbonate deposition. In the present study, a specific antibody against GAMP was raised and distribution of GAMP immunoreactivity was studied in crayfish gastrolith and exoskeleton. Localization of calcium carbonate in the exoskeleton, determined by silver nitrate staining and energy dispersive X-ray microanalysis, was compared with that of GAMP immunoreactivity. Crystalline forms of calcium carbonate were also determined. SDS-PAGE and Western blotting revealed that the gastrolith extract contained a major band which was GAMP-immunopositive and showed the same mobility as that of purified GAMP. The exoskeleton extract showed smear bands, but no immunoreaction was detected. By using immunohistochemistry, the anti-GAMP antiserum reacted almost uniformly with gastrolith matrix irrespective of gastrolith size. Epithelial cells of the gastrolith disc were also immunopositive. In the exoskeleton, exocuticle was strongly GAMP-immunopositive, whereas the endocuticle and membrane layer was slightly positive. The epicuticle was immunonegative. Calcium carbonate was detected in exocuticle, endocuticle and a part of the membrane layer, but not in the epicuticle. Thus, the distribution of GAMP immunoreactivity roughly corresponded with that of calcium carbonate. X-ray diffraction study showed that calcium carbonate in the gastrolith was amorphous, whereas that in the exoskeleton consisted of calcite crystals. These data indicate that a GAMP-immunoreactive substance is commonly distributed in the mineralized tissues of the crayfish, but may exist in a chemically different form in other tissues.

INTRODUCTION

The process by which organisms form minerals is termed biomineralization, and minerals formed by such organisms are referred to as biominerals. Biominerals ordinarily contain mineral components and a small amount of organic matrix to provide structural framework (Lowenstam, 1981). As a general scheme, organisms initially construct an organic framework followed by the active incorporation of mineral ions. Since mineral type, orientation of crystallographic axes, and microarchitecture are under genetic control, the organic matrix appears to have an essential role in the crystal formation process. Thus, precise understanding of the nature and function of organic matrices may provide keys by which biomineralization mechanisms may be clarified.

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The major crustacean biomineral consists of calcium carbonate deposited in the exoskeleton. Prior to ecdysis, crustaceans resorb calcium from the old exoskeleton and store it in a particular part of the body. In many crayfish species, the anterior part of the stomach is specifically differentiated into a pair of gastrolith discs, and a part of the resorbed calcium is stored as gastroliths in the form of calcium carbonate (Travis, 1960; Ueno, 1980). Gastroliths are digested after the molt and thus the liberated calcium is utilized in calcification of the new exoskeleton.

As a first step in understanding the biomineralization process in crayfish, we purified and characterized the major protein component of the insoluble organic matrix from the gastrolith of the crayfish, *Procambarus clarkii*, and called it gastrolith matrix protein (GAMP) (Ishii *et al.*, 1996, 1998). This protein has a molecular weight of about 50.5 kDa, and is strongly bound to chitin which makes up the insoluble framework of the gastrolith. It is probably acidic since a high proportion of glutamic acid residues were detected. It is thus possible that GAMP functions in the biomineralization process

in the crayfish, as do putative mineralization-related acidic matrix-proteins reported earlier in other invertebrate tissues (Weiner, 1983; Sucov *et al*., 1987; Iijima *et al*., 1991; Watabe *et al*., 1991; Kawaguchi and Watabe, 1993; Dauphin, 1996).

In the previous study, we cloned a cDNA encoding GAMP and found that RNA extracted from the epithelium of crayfish exoskeleton hybridized with the GAMP cDNA probe, suggesting that mineralized tissues of the crayfish commonly contain GAMP-like substance (Tsutsui *et al*., 1999). The present study was undertaken to further clarify the distribution of GAMP-like substance in the mineralized tissues of the crayfish. Antiserum against GAMP was raised, and extracts from the gastroliths and exoskeleton were analyzed by SDS-PAGE and Western blotting. The exoskeleton and the gastrolith discs containing gastroliths were then immunohistochemically examined. The localization of calcium carbonate in the exoskeleton, determined by silver nitrate staining and X-ray microanalysis, was compared with that of GAMP immunoreactivity. The crystalline forms of calcium carbonate in the gastrolith and exoskeleton were also analyzed with an X-ray diffractometer.

MATERIALS AND METHODS

Animals

Freshwater crayfish, *Procambarus clarkii*, were purchased from a local dealer and maintained in aquaria at room temperature. They were fed artificial fish or prawn diets.

Preparation of antiserum against gastrolith matrix protein (GAMP)

GAMP was isolated from gastroliths as described (Ishii *et al*., 1998). To obtain antiserum against GAMP, two New Zealand White rabbits were immunized. Each rabbit received a first subcutaneous injection at several different sites on the back with an emulsion of complete Freund's adjuvant (Wako, Osaka) and 100 μg of GAMP in 0.2 M phosphate buffered saline (pH 7.0) at a ratio of 1:1. Four additional injections were done in the same manner except that incomplete Freund's adjuvant (Wako) was used. Blood was collected from each rabbit 10 days after the last injection. The production and specificity of antibody was examined by a dot-blot assay on a Nytran nylon membrane (Schleicher and Schuell, Dassel, Germany) using a Western blot kit horseradish peroxidase system (Kirekegaad & Perry Laboratories Inc., USA). In this assay system, 0.1 μg of GAMP was detected by both antisera from two rabbits using a 10^4-fold dilution. Bovine serum albumin (Sigma, St. Louis, USA) was not detected by the antisera even with a 10^2-fold dilution. GAMP did not react with preimmune rabbit sera using a 10^2-fold dilution.

SDS-PAGE and Western blotting of extracts from gastroliths and exoskeleton

Samples from gastroliths were prepared by extraction of insoluble materials with 1% SDS containing 10 mM dithiothreitol at 100°C for 10 min after decalcification of gastroliths (Ishii *et al*., 1998). Samples from the carapace exoskeleton were prepared in the same manner as in the case of gastroliths. Both samples were concentrated by ultrafiltration using MOLCUT II LGC (Millipore, Bedford, USA). GAMP was purified as reported previously (Ishii *et al*., 1998). Samples amounts applied to each lane of the gel were derived from 17 mg of gastroliths and 20 mg of carapace exoskeleton. SDS-PAGE was performed with 7.5% gel essentially according to the method of Laemmli (1970), and the gel was stained with Coomasie Brilliant Blue (CBB) R-350 (Pharmacia Biotech).

Another set of samples for which amounts corresponded to one-tenth of the amounts used in CBB staining was applied to SDS-PAGE and blotted onto a PVDF membrane (Clear Blot Membrane-P, Atto, Tokyo). The membrane was incubated with 1% skim milk in 0.5 M NaCl containing 20 mM Tris-HCl (pH 7.5) at room temperature for 1 hr. After washing five times with the same buffer, the membrane was incubated with the anti-GAMP antiserum at 10^3-fold dilution with 1% skim milk in the same buffer. It was then washed five times with the buffer and incubated with goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad, Hercules, USA) at 3×10^3-fold dilution with 1% skim milk in the same buffer. After washing five times with the same buffer containing 0.1% Tween 20, it was stained with 3,3'-diaminobenzidine (DAB) (Funakoshi, Tokyo).

Immunohistochemistry

Crayfish were deeply anesthetized in an ice-cold water before dissecting out tissues. Gastrolith discs each containing a gastrolith were dissected from the crayfish 6–10 days after bilateral eyestalk ablation. Gastrolith size differed among individuals. Samples were divided into three groups depending on gastrolith size: small, middle, and large gastrolith groups. Exoskeleton pieces, including the carapace, abdomen and uropods, were dissected from crayfish which did not have gastroliths. Dissected tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight and stored in 70% ethanol at 4°C. Samples were then decalcified in a 0.2 M ethanolic triethylammonium EDTA solution (Scott and Kyffin, 1978) at 4°C, dehydrated in ethanol, and embedded in paraffin. Cross sections were cut at 4 μm and mounted on glass slides coated with BIOBOND (British Biocell International, Cardiff, UK).

Deparaffinized sections were first incubated for 30 min with a 0.6% H_2O_2 solution and subsequently with 2% normal goat serum solution for 30 min. The sections were then incubated overnight at 4°C with the anti-GAMP antiserum at 4×10^3-fold dilution. The localization of the antibody was visualized by the avidin-biotin-peroxidase complex (ABC) method (Hsu *et al*., 1981) using commercial reagents (Vectastain ABC-PO Kit, Vector Lab., Burlingame, USA) and DAB as a substrate. Sections were counter-stained with Mayer's hematoxylin (Wako). No significant staining was detected when normal rabbit serum was used instead of the antiserum.

Distribution of calcium carbonate in the exoskeleton

In order to compare the distribution of GAMP immunoreactivity with that of calcium carbonate in the exoskeleton, undecalcified sections of exoskeleton were prepared. Exoskeleton pieces, i.e., the carapace, abdomen and uropods were dissected from crayfish which did not have gastroliths and were fixed as described above. Samples were dehydrated and embedded in paraffin without decalcification. Cross sections were cut at 4 μm and mounted on glass slides coated with BIOBOND.

Deparaffinized sections were stained with 1% silver nitrate for 5 min under a fluorescent lamp to visualize the mineralized portions.

Some deparaffinized samples were dried, set onto brass stabs and sputter-coated with gold-palladium using an E-1030 ion sputter (Hitachi, Tokyo). They were examined with a Hitachi S-2150 scanning electron microscope (SEM), and energy dispersive X-ray microanalysis of calcium was done with an Emax-5770 (Horiba, Kyoto) coupled with SEM.

Crystalline form analysis

Gastroliths and carapace exoskeleton were powdered and their crystalline forms of calcium carbonate were analyzed with an X-ray diffractometer (MiniFlex, RIGAKU, Tokyo).

RESULTS

Electrophoretic patterns for extracts of gastrolith and cara-

pace exoskeleton together with purified GAMP are shown in Fig. 1. The gastrolith extract showed several bands which stained with CBB, among which the major band indicated the same mobility as GAMP. Western blotting showed that only this band was immunostained with the anti-GAMP antiserum. On the other hand, the exoskeleton extract gave smear bands with CBB staining but no signal on Western blotting.

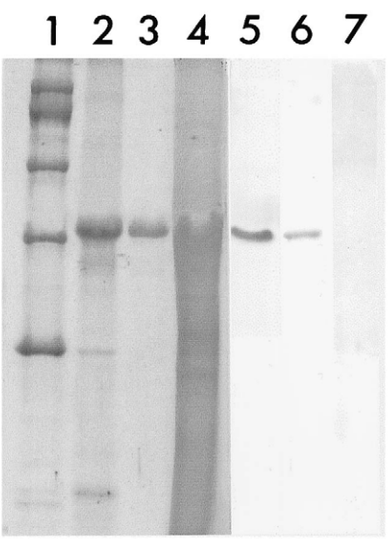
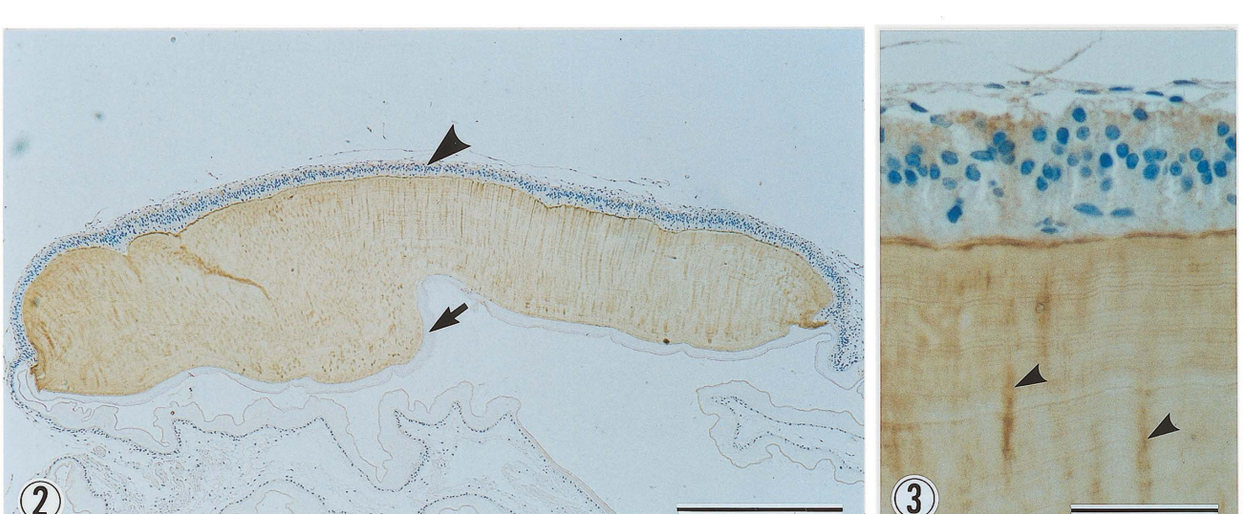


Fig. 1. SDS-PAGE and Western blotting stained with anti-gastrolith matrix protein (GAMP) antiserum for extracts from the gastroliths and carapace exoskeleton. Lanes 1–4, CBB staining; lanes 5–7, Western blotting using an anti-GAMP antiserum. Lane 1, molecular weight markers (Pharmacia Biotech; 212 kDa, 170 kDa, 116 kDa, 76 kDa and 53 kDa from the top); lanes 2 and 5, extracts from gastroliths; lanes 3 and 6, GAMP purified from gastroliths; lanes 4 and 7, extracts from the carapace exoskeleton.

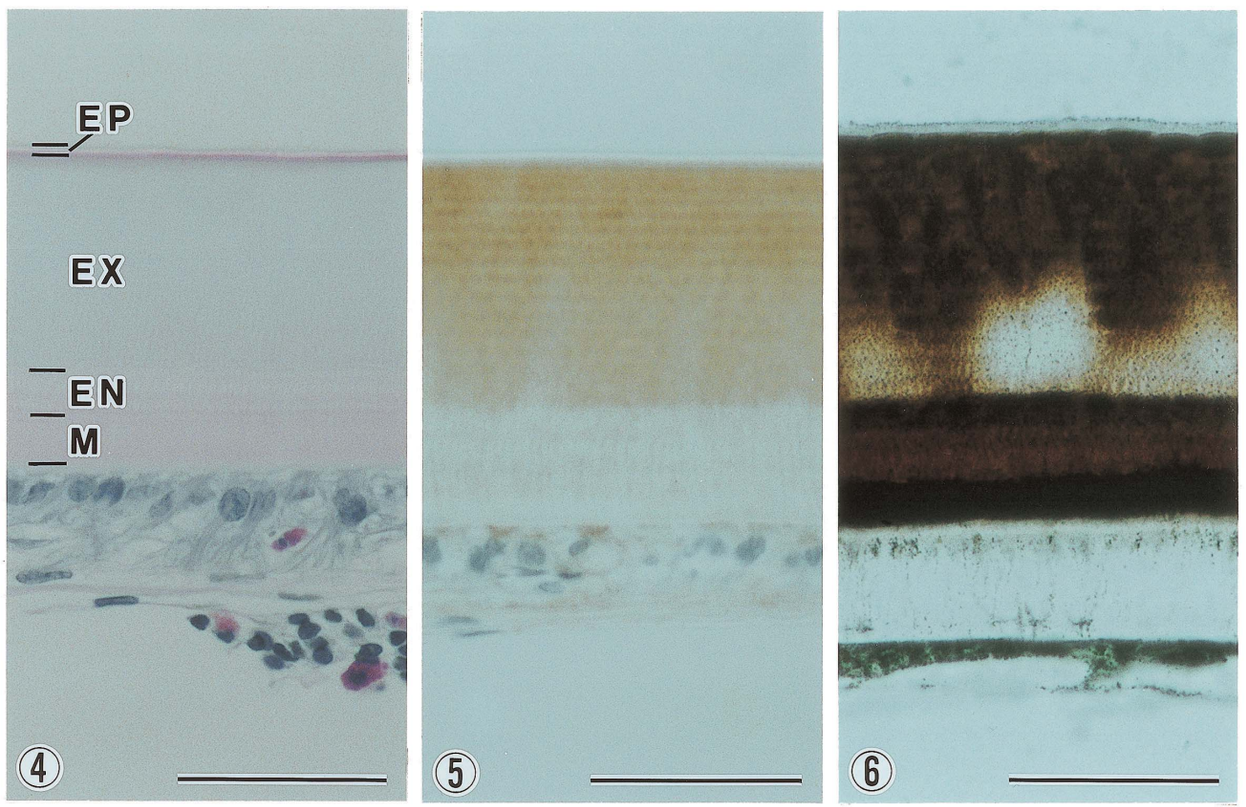
Fig. 2 and 3 show a section of gastrolith disc containing a small gastrolith. The gastrolith was formed between the epithelium and cuticle of the gastrolith disc (Fig. 2). The anti-GAMP antiserum reacted thoroughly with the gastrolith matrix (Fig. 2). Higher magnification (Fig. 3) showed a fine stratified structure, which consisted of layers showing a moderately or strongly positive reaction to the anti-GAMP antiserum. Strongly positive patches were dispersed within the matrix. Reactivity of gastrolith matrix against the antiserum was similar among small, middle and large gastrolith groups. Epithelial cells of the gastrolith disc consisted of mono-layered cylindrical cells positive to the antiserum (Fig. 3). Although the height of the epithelial cells decreased as the gastroliths grew larger, the reactivity of cells against the antiserum was almost constant. Epithelial cells of the stomach other than the gastrolith disc were cuboidal and showed no reaction to the antiserum (Fig. 2).

Fig. 4 is a photomicrograph of a section of abdominal exoskeleton stained with hematoxylin and eosin. The exoskeleton consisted of four cuticle layers; the outermost epicuticle, exocuticle, endocuticle and innermost membrane layer. Mono-layered epithelial cells were observed to be attached to the membrane layer.

The results of immunohistochemical staining against the anti-GAMP antiserum in the abdominal exoskeleton is shown in Fig. 5. The epicuticle showed no reaction toward the antiserum. Exocuticle was intensively positive toward the antiserum. Finely stratified layers composed of intensely and moderately positive layers were also visible. The endocuticle and membrane layer were slightly positive toward the antiserum. A positive reaction was observed in the apical part of the



Figs. 2 and 3. Light micrographs of the decalcified section of crayfish gastrolith disc stained with anti-gastrolith matrix protein (GAMP) antiserum. Fig. 2. Whole view of the gastrolith disc. The gastrolith was formed between the epithelium (arrowhead) and the cuticle (arrow) of the gastrolith disc. The anti-GAMP antiserum reacted thoroughly with the gastrolith matrix. Bar = 0.5 mm. Fig. 3. Higher magnification of the gastrolith epithelium and adjacent gastrolith matrix. Epithelial cells were positive to the antiserum. In the matrix, finely stratified layers and patches (arrowheads) showed strong positive reaction to the antiserum. Bar = 50 μm.



Figs. 4–6. Light micrographs of the cross section of crayfish abdominal exoskeleton. Bars = 50 μm. Fig. 4. Decalcified section stained with hematoxylin and eosin. The exoskeleton consisted of four cuticle layers; outermost epicuticle (EP), exocuticle (EX), endocuticle (EN) and innermost membrane layer (M). Fig. 5. Decalcified section stained with anti-gastrolith matrix protein (GAMP) antiserum. The exocuticle is positive, and the endocuticle and membrane layer are only slightly positive toward the antiserum. The epicuticle is immunonegative. Fig. 6. Undecalcified section stained with 1% silver nitrate. The exocuticle is positive but contains negative patches, whereas the endocuticle is intensely positive. The epicuticle and membrane layer are negative.

epithelial cells.

When an undecalcified section of abdominal exoskeleton was stained with 1% silver nitrate solution (Fig. 6), the epicuticle and membrane layer were negative, while the endocuticle was strongly positive. The exocuticle was also intensely stained, but it contained negative or slightly positive patches. The epithelial cell layer was also stained black.

The general morphology of the carapace and uropod exoskeleton was essentially the same as that of abdominal exoskeleton, although the thickness of each cuticle layer differed. The reactions of carapace and uropod exoskeleton against the anti-GAMP antiserum were similar to those of abdominal exoskeleton; the epicuticle was negative, the exocuticle was intensely positive, and the endocuticle and membrane layer were slightly positive. Reactions against the silver nitrate staining differed among the exoskeleton parts. In the carapace exoskeleton, the membrane layer in addition to the exo- and endocuticle stained intensely. In the uropods, on the other hand, the exocuticle was positive, but endocuticle was only weakly positive. The membrane layer was negative. The epicuticle was negative and exocuticle contained negative patches for all the exoskeleton parts.

In order to confirm that silver nitrate staining was based on a reaction with calcium carbonate, undecalcified sections were examined with SEM and energy dispersive X-ray microanalysis. Fig. 7 shows a typical SEM image of the section of abdominal exoskeleton and results of the X-ray microanalysis of calcium ($K\alpha_1$=3.690 keV) in the line-analysis mode. The exocuticle and endocuticle showed clear peaks of calcium whereas the epicuticle, membrane layer and epithelial cell layer showed background levels only.

Table 1 summarizes the distribution of GAMP immunoreactivity and calcium carbonate as determined by silver nitrate staining and X-ray microanalysis in the exoskeleton. The distribution of GAMP immunoreactivity roughly corresponded with that of calcium carbonate.

X-ray diffraction studies of the gastroliths and carapace exoskeleton showed that the calcium carbonate of the gastrolith existed in mainly an amorphous form, whereas that of exoskeleton consisted mostly of crystalline calcite.

DISCUSSION

The present immunohistochemical study demonstrated GAMP immunoreactivity not only in the gastroliths but also in all the three exoskeleton parts examined, indicating that

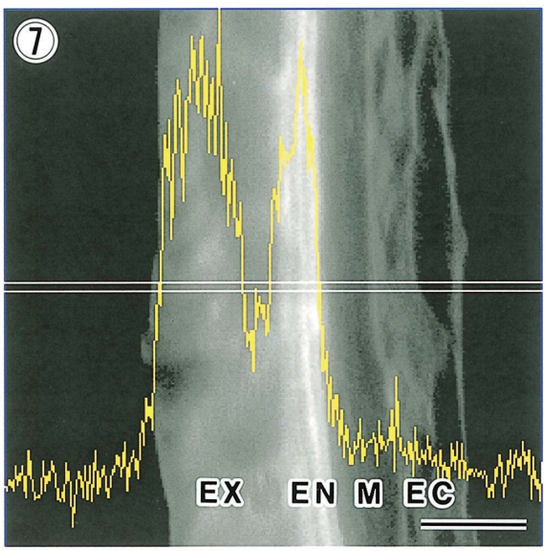


Fig. 7. A typical scanning electron microscope (SEM) image of a section of crayfish abdominal exoskeleton and results of X-ray microanalysis of calcium ($K\alpha_1$ = 3.690 keV, yellow line) in the line-analysis mode. The area between the two horizontal white lines was analyzed. Calcium is detected in the exocuticle (EX) and endocuticle (EN). The membrane layer (M) and epithelial cell layer (EC) do not contain significant levels of calcium compared with those of the background (glass slide). The epicuticle cannot be distinguished by SEM. Bar = 50 μm.

Table 1. Distribution of GAMP immunoreactivity (GAMP) and calcium carbonate ($CaCO_3$) as determined by silver nitrate staining and X-ray microanalysis in the exoskeleton of crayfish, *Procambarus clarkii*.

	GAMP	$CaCO_3$
Epicuticle	–	–
Exocuticle	++	±
Endocuticle	+	+
Membrane layer	+	±
Epithelial cell layer	+	–

–, Negative; ±, partly positive; +, positive; ++, intensely positive.

GAMP-immunoreactive substance is commonly contained in the mineralized tissues of the crayfish.

Western blotting of the gastrolith extract showed only one discrete band which had the same mobility as that of purified GAMP in SDS-PAGE, indicating that the antibody used in the present study reacted only with GAMP. Therefore, it can be concluded that the results obtained by immunohistochemistry for the gastroliths and the gastrolith disc reveal the distribution of GAMP itself. In the gastrolith, GAMP was detected throughout the matrix, irrespective of gastrolith size. Epithelial cells of the gastrolith disc also contain GAMP in all gastrolith size groups. These data indicate that GAMP is produced almost constantly during gastrolith formation, and is thus an essential component of the gastroliths. More detailed localization of GAMP in the gastrolith matrix was not consistent, as both strongly GAMP-immunopositive layers and patches were demonstrated. The significance of the microheterogenous distribution of GAMP in the gastrolith matrix is unknown at present.

Tsutsui *et al*. (1999) cloned a cDNA encoding GAMP and its deduced amino acid sequence showed a high proportion of glutamic acid residues, indicating that it is an acidic protein. They also demonstrated that GAMP inhibited calcium carbonate precipitation in an *in vitro* assay system. These results suggest the possibility that GAMP participates in gastrolith mineralization as do putative mineralization-related acidic proteins which have been reported in other invertebrate tissues (Weiner, 1983; Sucov *et al*., 1987; Iijima *et al*., 1991; Watabe *et al*., 1991; Kawaguchi and Watabe, 1993; Dauphin, 1996). In the present study, the gastroliths were shown by X-ray diffraction to be composed of amorphous calcium carbonate, suggesting that GAMP has function(s) in the precipitation of calcium carbonate in the amorphous form.

The present study also revealed that the exoskeleton contained a GAMP-immunoreactive substance. This is well in agreement with the results of Northern blot analysis which showed GAMP mRNA expression in the crayfish exoskeleton (Tsutsui *et al*., 1999). However, the GAMP-immunoreactive substance in the exoskeleton was not extractable when tissue was treated in the same manner as were gastroliths, as Western blot analysis of the exoskeleton extract showed no positive reaction against the anti-GAMP antiserum. These data suggest that, in the exoskeleton, the GAMP-immunoreactive substance exists in a form chemically different from GAMP in the gastroliths. It is possible that such a difference is related to the crystalline form of calcium carbonate in the tissue since gastrolith contains amorphous calcium carbonate, whereas the exoskeleton contains calcite crystals.

We compared the distribution of GAMP immunoreactivity with that of calcite crystals in the exoskeleton. In order to reveal the distribution of calcite crystals, we employed two histological methods, silver nitrate staining and X-ray microanalysis. Silver nitrate staining detects carbonate ion, while X-ray microanalysis detects calcium. The results obtained by the two methods in the cuticle layers were completely consistent. Therefore, it can be concluded that these methods visualized the distribution of calcite crystals. The present observations indicate that calcite crystals were most intensely deposited in the endocuticle. Intense deposition of calcite also occurred in exocuticle, but some patches seemed to remain uncalcified. Calcite crystals were deposited highly in the membrane layer of carapace exoskeleton, but to no extent in any other part of the exoskeleton. On the other hand, GAMP immunoreactivity was demonstrated most intensely in the exocuticle, and faintly in the endocuticle and membrane layer in all exoskeleton parts studied. The epicuticle, which contains no calcite crystals, was immunonegative. Thus, the distribution of GAMP immunoreactivity roughly corresponded with that of calcite crystals, implying that GAMP-immunoreactive substance in the exoskeleton may be related to calcite crystallization. However, the intensity of GAMP-immunoreactivity was not proportional to the amount of calcium carbonate in each cuticle layer. Further detailed studies are required to examine the function(s) of GAMP and the GAMP-immunore-

active substances in the crayfish mineralization process. It is also important to characterize the GAMP-immunoreactive substance present in the exoskeleton.

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