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Control of CaCO₃ Crystal Growth by the Acidic Proteinaceous Fraction of Calcifying Marine Organisms: An *In Vitro* Study of Biomineralization

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1. Introduction

Only little is known about the early stages of CaCO₃ crystallization (Pouget et al. 2009; Gebauer, Volkel, and Colfen 2008), though this mineral has been studied for more than a century now. Identified precursor phases are amorphous calcium carbonate (ACC) in bio- (Addadi, Raz, and Weiner 2003; Weiner et al. 2003) and biomimetic mineralization. Crystal nucleation and biomineralization processes in organisms occur through a sophisticated regulation of internal chemistry that departs significantly from the “constant ionic medium” of seawater (Falini et al. 1996; Addadi et al. 2006; Rahman and Oomori 2009). Magnesium ions are mainly responsible for controlling the kinetics and thermodynamics of calcium carbonate precipitation, especially inhibition of the calcite formation (Davis, Dove, and De Yoreo 2000). The precipitation of calcite at ambient temperature is both thermodynamically and kinetically favored in solutions containing low amounts of magnesium ions. Very recently, it is reported that although Mg²⁺ is influential in producing aragonite in the crystallization process, acidic macromolecules produced calcite crystals in soft corals even in the presence of high Mg²⁺ ions (Rahman, Oomori, and Worheide 2011; Rahman and Oomori 2009).

In the crystallization processes, nucleation is considered to take place in a solution of ions or molecules exceeding a critical supersaturation, leading to the nucleation of the new phase (Addadi, Weiner, and Geva 2001). The growth of nucleated particles and crystals is then considered to take place via addition of single ions or molecules. The role of additives, which modify crystal growth (Rahman and Oomori 2008; Rahman et al. 2011), is restricted in such a view: they can either bind ions or interact with the crystal (Addadi, Berman, Moradianoldak, et al. 1989; Addadi, Weiner, and Geva 2001). It is reported that the crystals

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grown were completely inhibited and no crystals were observed when a high amount of protein (containing 105 mg/6 ml) was added in the reaction vessel (Rahman and Oomori 2008). The role of additives is, however, still hard to attribute, and empirical control of morphology is the rule, not the exception. This is caused by the multiple roles of additives in such processes, which in addition depends on concentrations and other experimental conditions (Addadi, Berman, Oldak, et al. 1989; Addadi, Weiner, and Geva 2001). A possible quantification, at least a classification of all the different interactions, is eagerly needed, but simple tools to do so are not currently fully known, except few reports (Meldrum and Colfen 2008; Gebauer, Volkel, and Colfen 2008; Rahman, Oomori, and Worheide 2011).

This review aims at opening the pathway to such systematization, here exemplified for calcium carbonate as a model system of complex crystallization. The choice is based on relevance: calcium carbonate is not only of great industrial importance, the major source of water hardness, and the most abundant biominerals, but also one of the most frequently studied minerals, with great scientific relevance in biomineralization and geosciences. Scale formation is also a substantial issue in daily life, industry, and technology, rendering the addition of scale inhibitors to laundry detergents, household cleaners, and also in many industrial applications unavoidable.

2. Mollusks

Biominerals of marine organisms, especially mollusk shells, generally contain unusually acidic proteins (Takeuchi et al. 2008). These proteins are believed to function in crystal nucleation and inhibition. Takeuchi et al (2008) identified an unusually acidic protein Aspein from the pearl oyster *Pinctada fucata*. They showed that Aspein can control the CaCO₃ polymorph (calcite/aragonite) in vitro (**Fig. 1**). Their results suggest that Aspein is involved in the specific calcite formation in the prismatic layer and the experiments using truncated Aspein demonstrated that the aspartic acid rich domain is crucial for the calcite precipitation. Mollusks, like many other mineralizing organisms, including the vertebrates, first isolate their environment of mineral formation from the outside world (Simkiss 1989). Mollusks use a highly cross-linked protein layer (periostracum) and the epithelial cells of the mantle, the organ directly responsible for shell formation. They then elaborate a matrix within this space comprising various macromolecules (Weiner and Hood 1975; Weiner 1979). This matrix is the framework in which mineral forms (Miyamoto et al. 1996). The major components of the matrix are the polysaccharide beta-chitin, a relatively hydrophobic silk protein, and a complex assemblage of hydrophilic proteins, many of which are unusually rich in aspartic acid (Amos and Evans 2009; Tsukamoto, Sarashina, and Endo 2004; Lowenstam 1989; Samata et al. 1999; Gotliv et al. 2005). The final stage of the process is the formation of the mineral itself within the matrix. Some of the acidic proteins are also occluded within the mineral phase as it forms (Suzuki et al. 2009). The mineral in mature mollusk shells is most often aragonite, sometimes calcite, and in certain taxa, the same shell may have layers of calcite and layers of aragonite (Simkiss 1989; Lowenstam 1989; Suzuki et al. 2009).

Each mineralized tissue contains tens of different macromolecules, many of which are not unique to the mineralized tissue, but can be found in other tissues as well (Arias 2007; Veis 2003; Wilt 2007). There is however one group of glycoproteins that is unique to many

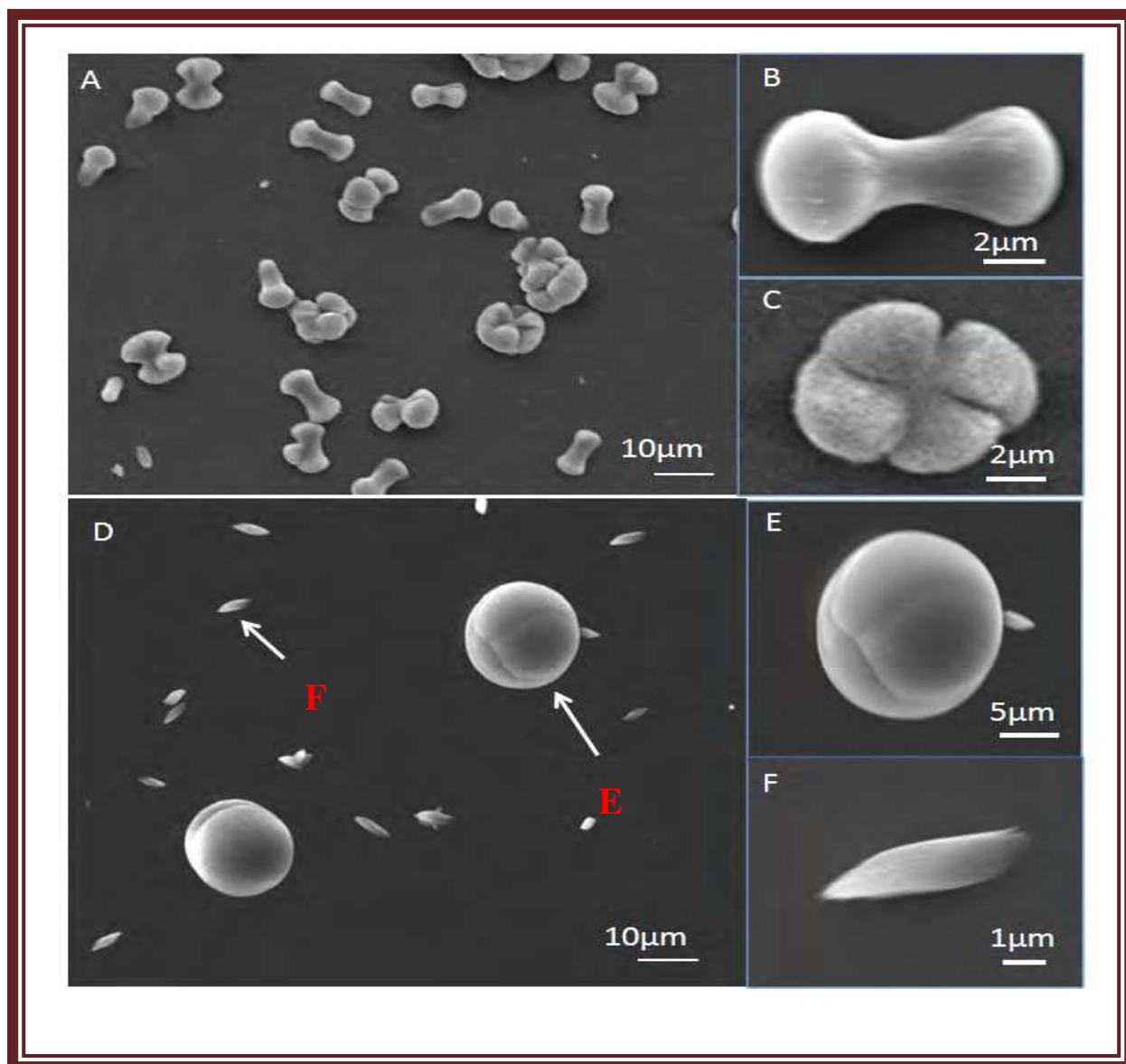


Fig. 1. SEM images of CaCO₃ crystals. (A–C) Crystals grown in the presence of Aspein at 10 μg/ml. Dumbbell-like crystals (A, B) are formed. (C) Fused dumbbells are also observed. Under this condition, all the crystals formed are calcite. (D–F) Crystals grown in the presence of Aspein at 2 μg/ml. A spherical crystal (aragonite) is indicated by an arrow in (D) and enlarged in (E). A polyhedral crystal (calcite) is indicated by an arrowhead in (D) and enlarged in (F). From Takeuchi et al. 2008 (with permission)

mineralized tissues. These are proteins that are rich in acidic amino acids, usually aspartic acid (Marin 2007; Weiner 1979). Only a few of these proteins have been sequenced (Weiner 1979; Suzuki et al. 2009), as there are many technical problems in purifying and characterizing such highly charged molecules. Recently, Suzuki, et al. (2009) identified an acidic matrix protein (Pif) in the pearl oyster *Pinctada fucata* that specifically binds to aragonite crystals. The results from immunolocalization, a knockdown experiment that used RNA interference and *in vitro* calcium carbonate crystallization studies strongly indicate that Pif regulates nacre formation. Others from mollusks have complex domain

structures (Sarashina and Endo 1998), including in one case long stretches of polyaspartic acid (Gotliv et al. 2005). These proteins are thought to be the active components of the mineralization process (Marin 2007). They are relatively well investigated in mollusk shell formation. Some are thought to be involved in the formation of the disordered precursor phase, others in the crystal nucleation and growth processes, and others are located within the crystal itself where at least in the case of calcite, they change the materials properties of the crystal (Addadi et al. 2006; Berman et al. 1993; Berman et al. 1990; Nudelman et al. 2007). Such structural proteins do not readily crystallize and the crystal structures of these mineralizing proteins are not yet known. In fact, it seems highly unlikely that they will form crystals, and therefore 3-dimensional structural information may need to be obtained by high resolution cryo-tomography in vitrified ice and/or by solution NMR. Many mineralized tissues fulfill mechanical functions because the presence of mineral causes the tissue to be relatively stiff. Thus understanding the relations between and function often refers to mechanical functions (Addadi and Geva 2003; Addadi and Weiner 1997; Addadi et al. 1995), this is not easy.

Recently, Marie and coworkers (Marie et al. 2008) extracted matrix proteins from a freshwater mussel (*Unio pictorum*) and they found that the nacre-soluble matrix exhibits a carbonic anhydrase activity, an important function in calcification processes. In this study, the shell acid-soluble matrices of prismatic and nacreous layers were prepared. Among the proteins extracted from the shell of *U. pictorum*, P95 was a major component, specific to the nacre acidic soluble matrix (ASM). It was absent in the prismatic layer, unlike the other discrete components. This suggests that P95 might play an important function in controlling, inter alia, the building of nacre during shell formation. P95 is a glycoprotein, the acidity of which is entirely conveyed by its glycosyl moieties, consisting of acidic and sulfated polysaccharides. In its amino acid composition, P95 presents the “signature” of an acidic protein, because of its high Asx and Glx residue content (Weiner 1979). Furthermore, its glycosyl moiety, consisting of sulfated polysaccharides, is involved in calcium binding. To estimate the effects of P95 on the morphology of calcium carbonate crystals, they examined crystals obtained in the presence of P95 by SEM and compared the results with those of a positive control experiment performed with the whole nacre ASM (Marie et al. 2008). Zhang et al. (Zhang, Xie, et al. 2006; Zhang, Li, et al. 2006) isolated a novel matrix protein, designated as p10 from the nacreous layer of pearl oyster (*Pinctada fucata*) by reverse-phase high-performance liquid chromatography. In vitro crystallization experiments showed that p10 could accelerate the nucleation of calcium carbonate crystals and induce aragonite formation, suggesting that it might play a key role in nacre biomineralization.

3. Vertebrates

One of the best characterized is also the one first identified (Veis 1967), namely phosphophorin extracted from vertebrate teeth (Veis 2003). Often the structures involved, especially those of the vertebrates, are not only hierarchical but also graded – they change in a systematic manner from one location to another (Tesch et al. 2001). One approach for gaining insight into structure function relations in such tissues is to take advantage of the array of surface probe instruments that can provide information on both materials properties and structure at the nanometer level. This indeed has proved to be a powerful

approach (Kinney et al. 1999; Moradian-Oldak et al. 2000). A problem with these methods is that it is difficult to integrate the localized information into understanding how a whole organ such as a bone or tooth functions. An alternative approach is to monitor how whole organs deform under load, by mapping the displacements at the nanometer level, and in this way relate them to the structure. This can be done using various optical metrology methods (Shahar 2007). One particularly promising method is electronic speckle pattern interferometry (ESPI), that provides nano-scale deformation information on irregular surfaces even when the object is under water, which is essential for the study of biological tissues (Zaslansky 2006). It is still however a real challenge to integrate structural information at the millimeter, micrometer and nanometer scales and relate this to mechanical properties that are of course the product of all these structures 'working' synergistically. A significant achievement in this regard, is the study by Gupta et al (Gupta et al. 2006) on bone structure-function relations.

An unexpected discovery in the vertebrate biomineralization field was that many tissues of mice in which a minor bone protein, called Matrix Gla Protein (MGP) was removed, spontaneously mineralized (Luo et al. 1997). (Gla or γ -carboxyglutamic acid is a most unusual amino acid that resembles glutamic acid, except that it has two carboxylate groups). Clearly one function of MGP is to prevent such catastrophic mineralization. It was also shown that the common serum protein, fetuin-A, has a similar function (Heiss 2002). The calcium phosphate mineral in bones and teeth, carbonated hydroxyapatite, is a relatively insoluble mineral, and there is sufficient calcium and phosphate in vertebrate tissues for them to be saturated with respect to bone mineral. Thus, in the absence of crystal inhibitors, tissues spontaneously mineralize. This led to the interesting proposal that removal of inhibitors is the basic requirement for bone to mineralize (Murshed 2007).

4. Corals

The mechanism by which biomineralization occurs in corals is poorly known. It is reported that corals are composed of calcium carbonate in an organic matrix (Watanabe et al. 2003; Rahman, Oomori, and Worheide 2011; Rahman and Oomori 2008; Rahman et al. 2006; Rahman and Isa 2005; Rahman, Isa, and Uehara 2005; Rahman and Oomori 2008; Fukuda et al. 2003). The organic matrix is formed prior to mineralization, and it has been suggested that some components of the matrix protein may serve as a template for mineral deposition (D'Souza 1999; Weiner and Hood 1975). Recent reports have focused on the characterization of proteins in the soluble matrix of soft coral sclerites (Rahman, Oomori, and Worheide 2011; Rahman and Oomori 2009, 2009, 2008; Rahman, Isa, and Uehara 2005; Rahman et al. 2006; Rahman 2008; Rahman et al. 2011; Rahman and Isa 2005) and stony corals (Watanabe et al. 2003). Also on the control of the morphology and the chemical composition of calcitic bio-crystals in some precious corals have been reported (Dauphin 2006). Compared to the information available on stony corals, molluscs, calcareous algae, and other matrices (Miyamoto et al. 1996; Linde, Lussi, and Crenshaw 1989; Marin, de Groot, and Westbroek 2003; Watanabe et al. 2003; Falini et al. 1996), very little is known regarding matrix components of corals. There are two important features of biomineralization. First, a relatively inert structural frame is built from insoluble macromolecules (hydrophobic proteins, chitin). Second, acidic proteins (rich in aspartic acid, and often in association with sulfated polysaccharides) are assembled on the framework (Mann 1993). It remains important to better understand the role of these matrix

fractions in calcification. This can be accomplished by several approaches such as determining how they influence the morphology and composition of the mineral formed.

From the last few years (Rahman et al, 2006, 2009, Watanabe et al 2003), acidic proteins were purified from the organic matrices of corals. In the present review, the crystallization of corals in the presence of these proteins was discussed and compared with other macromolecules. Crystallization plays a key role in the bio-calcification process and ultimately in the growth of coral skeletons. One widely used approach for studying the functions of these acidic proteins is to examine their effect on crystal growth, *in vitro*. Combinations of matrix components have been used to detect a collaborative effect (Termine et al. 1981), and the ability of demineralized matrix to induce crystal nucleation has been examined (Rahman, Oomori, and Worheide 2011). The objective was to understand the principles that govern these interactions and to gain insight into the mechanisms by which these matrix constituents regulate crystal growth *in vivo*. The major polymorphisms involved in CaCO_3 crystallization of marine organisms were identified and subsequently, the functions of specific organic matrix proteins in the bio-calcification process were determined.

The similar results were found in the soft corals sclerites (**Fig. 2**). To investigate the influence of matrix proteins on calcium carbonate crystals *in vitro*, the morphology of crystals grown with or without any protein was observed under SEM and XRD by Rahman et al. (**Fig. 3**). Two crystallization solutions were prepared, one that induces calcite (calcitic crystallization solution) and one that induces aragonite (aragonitic crystallization solution). The calcitic solution was a supersaturated solution of $\text{Ca}(\text{HCO}_3)_2$ prepared by purging a stirred aqueous suspension of CaCO_3 with carbon dioxide.

The crystals grown in the absence of protein exhibited the characteristic rhombohedral morphology of calcite (**Fig. 2A**). However, crystals grown in the presence of matrix protein showed an interesting phenomenon. In comparison to the control measurement, crystal growth density was lower when a high amount of matrix (containing 45 $\mu\text{g}/6\text{ml}$ protein) was added into the reaction vessel (**Fig. 2B**). The morphology of crystals was also affected in the presence of 45 μg of protein (see enlarged image of the boxed part in **Fig. 2C**). Subsequently, when 1/10 of the amount of matrix (containing 4.5 $\mu\text{g}/6\text{ml}$ protein) was added to the solutions at the same time, crystal growth increased and a few crystals were affected (**Fig. 2D**). No crystals were observed under SEM when an even higher amount of protein (containing 105 $\mu\text{g}/6\text{ml}$ protein) was added in the reaction vessel (**Fig. 2E**). These results demonstrate the regulation of crystal growth by protein in a CaCO_3 system in alcyonarian corals, specifically the inhibition of crystal growth. This study concurred with the similar works recently conducted by Takeuchi and coworkers (Takeuchi et al. 2008; Suzuki et al. 2009).

Further, precipitation of CaCO_3 was simulated *in vitro* in the presence of both soluble and insoluble organic matrix proteins of sclerites at the ratio of 29:60 mol% (**Fig. 3**). In every experiment, influence of proteins with the precipitated crystals was examined by XRD. **Table 1** summarizes the percentage of aspartic acid and other amino acids in the soluble and insoluble matrix proteins used for crystallization. **Figure 3** shows the CaCO_3 crystal growth and morphology in the absence or presence of matrix proteins. The influence of Mg^{2+} on CaCO_3 polymorphism was also studied. Without Mg^{2+} , typical rhombohedral calcite crystals were generated (**Fig. 3A, B and L**). In the presence of Mg^{2+} (50 mM), large needle-like crystals were preferentially formed (**Fig. 3C, D and M**). **Fig. 3 (E-K)** shows the SEM images

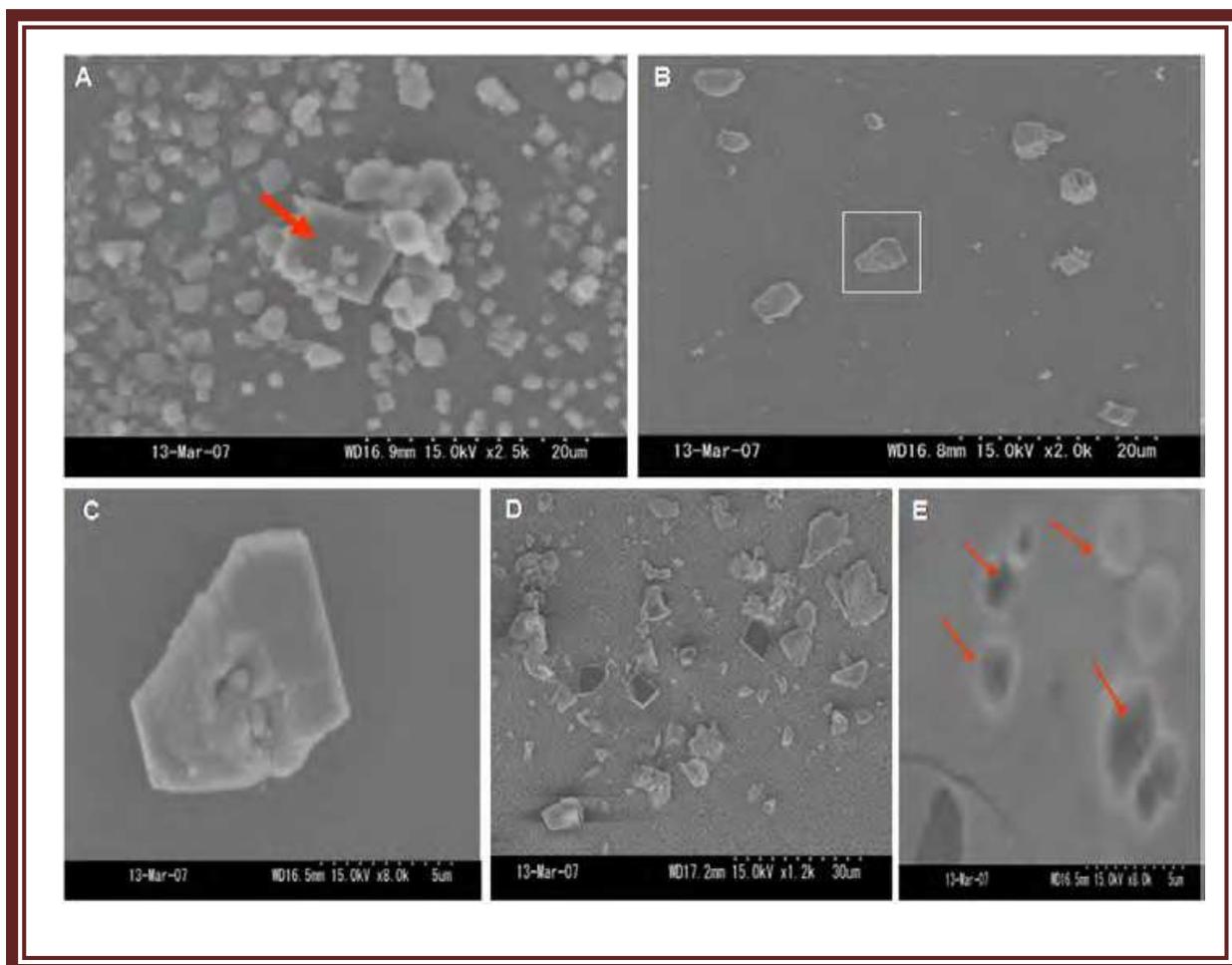


Fig. 2. SEM observation of crystals in the presence or absence of matrix proteins isolated from the calcitic sclerites. (A) Calcite rhombohedrons grown in the absence of matrix proteins, the arrow indicates the {104} face of the crystals. (B) Calcite crystals grown in the presence of matrix proteins (protein concentration 45 mg/6 ml). (C) Enlarged image of the boxed part in (B). (D) Calcite crystals grown in the presence of matrix proteins (protein concentration 4.5 mg/6 ml). (E) Result of a crystallization experiment that was carried out by the same procedure as in (B) and (D) but in the presence of high amount matrix proteins (containing 105 mg/6 ml). There is no mineral deposition (also at higher magnification), i.e., only the glass spot or very tiny crystals are seen (arrows), indicating that the crystallization is completely inhibited by the high content of matrix proteins. Scale bars: A= 20 μm ; B=20 μm ; C= 5 μm ; D=30 μm ; E=5 μm . From Rahman and Oomori 2008.

of CaCO₃ crystals grown in the presence of matrix protein, at a lower and a higher concentration, 0.5 and 1.4 $\mu\text{g}/\text{mL}$, respectively. At a concentration of 0.5 $\mu\text{g}/\text{mL}$, a number of spherical crystals (aragonite) remained (**Fig. 3E indicated by an arrowhead, F**), and some polyhedral and round calcite crystals were exclusively induced (**Fig. 3E indicated by arrows, G, H**). The XRD measurements (**Fig.3N**) demonstrated that the both aragonite and calcite crystals are available in this experiment. The higher concentration of matrix proteins (1.4 $\mu\text{g}/\text{mL}$) showed a high intensity of rhombohedral calcite crystals (**Fig. 3I, J, K**) without any aragonite formation. Although the growth of crystals was inhibited at high

concentrations of proteins, all remaining aragonites formed by Mg^{2+} (50 mM) were transformed into calcites (**Fig. 3I and enlarged view indicated by arrows in J, K**). The XRD measurements proved that all crystals formed under these conditions were calcites (**Fig. 3O**). These observations strongly suggest that the acidic matrix proteins are the key components in forming calcite crystals in biocalcification.

From our observation, the density of nucleation sites was lower when the crystals were grown with a mixture of soluble and insoluble matrix proteins in which the aspartic acid content of the insoluble matrix was 60mol% (**Fig. 3I**). Also, soft corals have special characters because the organic matrices themselves are highly aspartic acid-rich proteins (Rahman and Oomori 2009). In addition, previous studies on molluscan shells indicate that acidic amino acid residues may actually inhibit crystal nucleation (Wilbur 1982). The present review reveals that both matrix fractions of some marine organisms were enriched in aspartic acid proteins (Weiner 1979; Takeuchi et al. 2008; Rahman and Oomori 2009); the especially high aspartic acid content of insoluble organic matrix proteins may regulate the crystal growth and morphology (Suzuki et al. 2009; Rahman et al. 2006; Rahman 2008), or could play a key role in crystal nucleation induction (Addadi et al. 2006; Addadi, Raz, and Weiner 2003; Addadi et al. 1995; Addadi, Berman, Oldak, et al. 1989).

Amino acid	Soluble fraction		Insoluble fraction	
	Molecular weight of residues	mol%	Molecular weight of residues	mol%
Cys	151.14	0.41	151.14	0.25
Asx (Asp+Asn)	115.09	29.35	115.09	60.92
Thr	101.10	5.18	101.10	1.57
Ser	87.08	4.61	87.08	1.52
Glx (Glu+Gln)	129.11	9.63	129.11	5.30
Gly	57.05	10.37	57.05	9.40
Ala	71.08	11.37	71.08	12.87
Val	99.13	5.06	99.13	1.26
1/2- Cys	103.14	1.85	103.14	0.55
Met	131.20	0.82	131.20	0.17
Ile	113.16	2.99	113.16	0.74
Leu	113.16	3.56	113.16	1.05
Tyr	163.17	0.80	163.17	0.32
Phe	147.17	1.93	147.17	0.51
Lys	128.17	2.66	128.17	0.79
His	137.14	0.78	137.14	0.29
Arg	156.19	1.89	156.19	0.92
Trp	186.20	0.00	186.20	0.00
Pro	97.11	6.73	97.11	1.60
Protein Total		100.00		100.00

Table 1. Amino acid composition of the protein in the total soluble and insoluble fractions from the calcitic sclerites of *S. polydactyla*. From Rahman and Oomori 2009

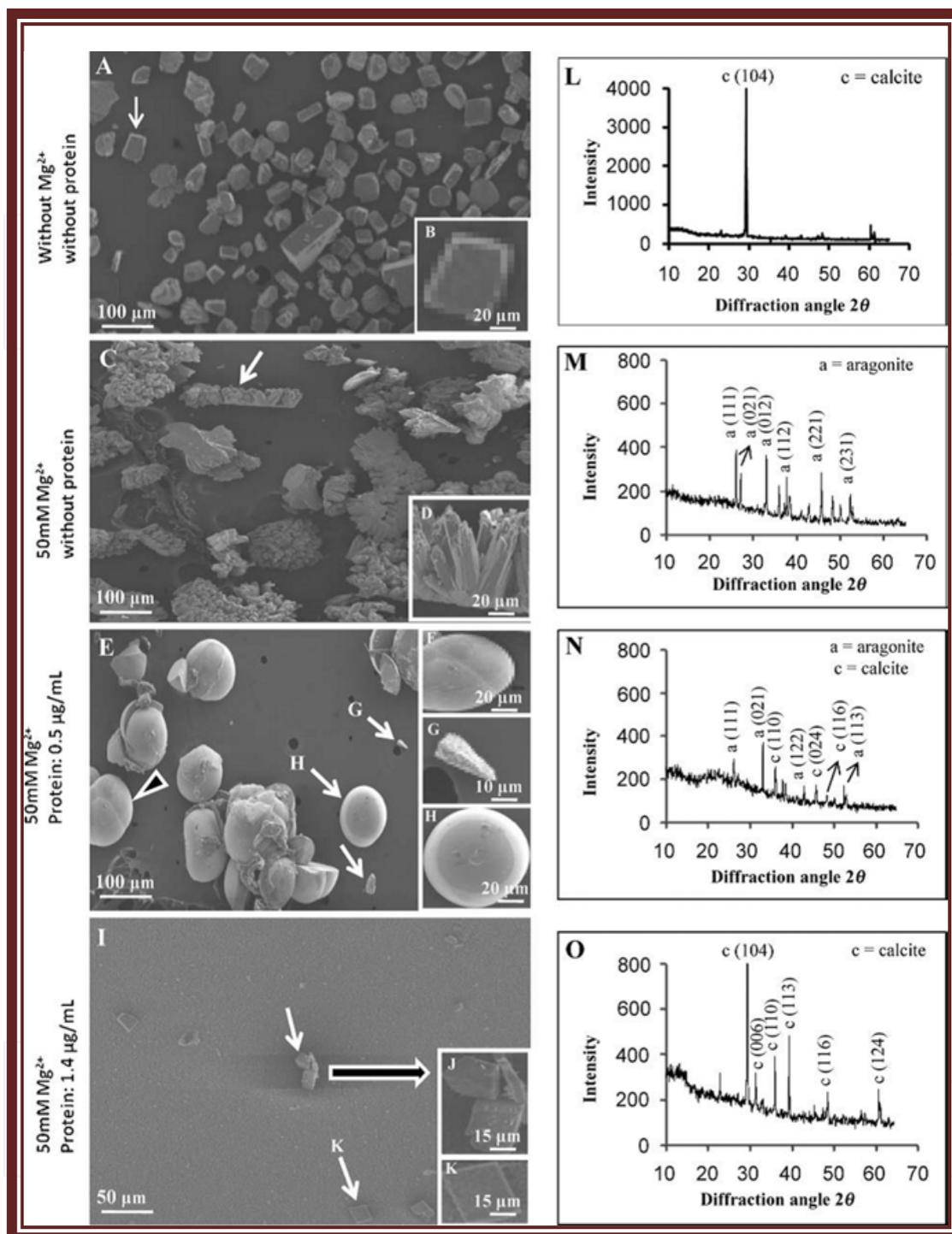


Fig. 3. SEM images of CaCO₃ crystals growth *in vitro* and their XRD diffractions. A mixture of soluble and insoluble organic matrix proteins (insoluble = 60% aspartic acid; soluble = 29% aspartic acid) isolated from the calcitic sclerites was used in crystallization experiments. (A, B) Crystals grown without proteins in the absence of Mg²⁺ shows rhombohedral calcite crystals. (C, D) Crystals grown without proteins in the presence of Mg²⁺ shows needle-like crystals (aragonite). (E-H) Crystals grown in the presence of proteins (0.5 µg/mL) with Mg²⁺ (50 mM). A number of spherical crystals (aragonite) remained (indicated by an arrowhead in E and enlarged in F), and some polyhedral and round calcite crystals were exclusively induced

(indicated by arrows in E and enlarged in G, and H). The polymorphs of CaCO₃ were identified both by XRD and Raman microprobe analysis (see Figure 3 for Raman data). (I-K) Crystals grown in the presence of proteins (1.4 μg/mL) with Mg²⁺ (50 mM). Under these conditions, all the crystals formed are calcites (arrows in I and enlarged in J and K). (L-O) Verification of crystals formed in these experiments by XRD. From Rahman and Oomori 2009

5. Concluding remarks

The overall aim of this review is to better understand the function of the acidic proteins in the matrices of marine organisms during mineral formation by using an *in vitro* analysis approach. Since it is difficult to understand biological systems based on *in vivo* analyses of control and regulation processes, the actions of additives such as acidic proteins, which are mostly available in calcifying marine organisms, have been discussed here based on *in vitro* analyses. *In vitro* experiments reported in the literature reveal that acidic proteins are primarily responsible for the control of CaCO₃ polymorphisms in mollusk shells and corals. In this paper, we used data from *in vitro* experiments with mollusk shells and corals, since these marine organisms contain high concentrations of acidic proteinaceous fractions. We compared the data from vertebrate studies to evaluate the differences between calcifying marine organisms (mollusk shells and corals). However, because of the different characterization of proteinaceous fractions in vertebrates, polymorphism control and regulation processes are completely different among invertebrate calcifying marine organisms. We conclude that there is potential for the control of CaCO₃ precipitation in calcifying marine organisms, which contain a high content of proteinaceous fractions. Our review suggests that the acidic matrix proteins in calcifying marine organisms are a specialized nucleating sheet that governs the nucleation of highly-oriented calcite or aragonite crystals and, thus, lead to a finer crystallization of CaCO₃.

6. Acknowledgments

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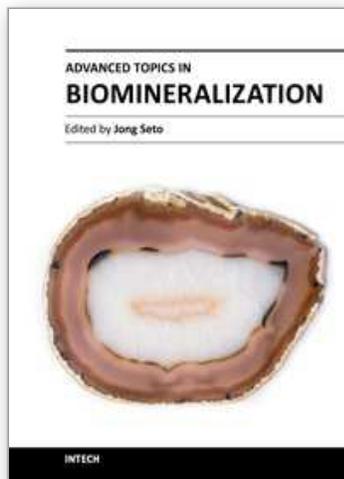
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