International Journal of Chemical, Materials and Biomolecular Sciences
ISSN: 2415-6620
Vol:3, No:8, 2009

Influence of Cell-free Proteins in the Nucleation of $CaCO_3$ Crystals in Calcified Endoskeleton

M. Azizur Rahman and Tamotsu Oomori

Abstract—Calcite and aragonite are the two common polymorphs of $CaCO_3$ observed as biominerals. It is universal that the sea water contents a high Mg^{2+} (50mM) relative to Ca^{2+} (10mM). *In vivo* crystallization, Mg^{2+} inhibits calcite formation. For this reason, stony corals skeleton may be formed only aragonite crystals in the biocalcification. It is special in case of soft corals of which formed only calcite crystal; however, this interesting phenomenon, still uncharacterized in the marine environment, has been explored in this study using newly purified cell-free proteins isolated from the endoskeletal sclerites of soft coral. By recording the decline of pH *in vitro*, the control of $CaCO_3$ nucleation and crystal growth by the cell-free proteins was revealed. Using Atomic Force Microscope, here we find that these endoskeletal cell-free proteins significantly design the morphological shape in the molecular-scale kinetics of crystal formation and those proteins act as surfactants to promote ion attachment at calcite steps.

***Keywords*—**Biomineralization, Calcite, Cell-free protein, Soft coral

I. Introduction

SOFT corals form an important part of the coral ecosystem. They occur commonly in all reef habitats, and provide economic and environmental services, such as sources of natural beauty and recreation, food, jobs, chemicals, pharmaceuticals, and shoreline protection. Soft corals can also be a source of useful chemicals, such as antibiotics or other drugs. Research is now being done on a method to encourage bone growth in humans, similar to how the soft corals secrete sclerites (endoskeletons). Thus, understanding the health of soft corals and how to utilize them for human food, medicine, and other ecosystem function-related questions in the ocean, is essential.

The key components of soft corals are tiny endoskeletal spicules of calcium carbonate called "sclerites." They are distributed throughout the mesoglea of the entire colony, increasing in number and size toward the base of the soft corals. Soft corals growth and calcification are completely controlled by sclerites [1]. It is generally known that a biological system has a unique ability to control crystal polymorph, structure, orientation, and hierarchical structure of inorganic phases. The crystals, which are an integral part of this structure, are composed of calcium carbonate. Their presence in the skeletons of invertebrates and vertebrates is particularly widespread. The crystals often have uniform size, oriented crystallographic axes, and adopt sizes and shapes that are quite different from those found in their non-biological counterparts. These properties indicate that the crystals form under well-controlled conditions [2]. The matrix macromolecules (proteinaceous components) are closely associated with the mineral phase and are thought to regulate crystal growth [3]. These macromolecules are acidic [3- 6].

In the present study, we conducted research with a soft coral, *Lobophytum crassum*, as a model of a calcifying marine organism. We purified cell-free proteins from sclerites and these proteins components have calcium-binding properties [7] and highly acidic [8-10]. Two functions have been suggested for such proteins: (i) the calcium associated with them may be involved in the initiation of nucleation [11, 12], and (ii) the protein may inhibit and thus regulate crystals growth [12, 13], possibly by binding to the growing centers of mineral [13]. The studies presented here demonstrate an effect of cell-free soluble matrix proteins on the rate of $CaCO_3$ deposition from the model solution and their interaction with the structure of crystals. 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.

II. Materials and Methods

A. Sample Collection and Preparation

Sclerites were separated from the coral colony (*Lobophytum crassum)* according to the mechanical and chemical treatments followed by Rahman *et al*. [14]. Briefly, the collected sclerites were stirred vigorously in 1 M NaOH for 2 h and subsequently in 1% NaClO solution for 2 h to remove the fleshy tissues and debris. Treated samples were washed under tap water until the sclerites were completely cleaned. Finally, samples were washed with distilled water (five times) and ultrapure water (MilliQ) to remove unwanted substances.

B. In vitro Crystallization

In vitro Crystallization experiments were carried out by adding matrix proteins according to the methods of Rahman et al. [15] and Borman et al. [16]. Crystals were grown from 3 ml of 20 mM $CaCl_2$ and 3 ml of 20 mM $NaHCO_3$ solution at pH 8.7. Each experiment was repeated every 30 minutes after

M. Azizur Rahman is with the University of the Ryukyus, Dept. of Chemistry, 1 Senbaru, Nishihara-cho, Okinawa, Japan (corresponding author) Phone: +81-90-4471-9291; fax: +81-98-895-8565; e-mail: azizur31@yahoo.com, g083001@sci.u-ryukyu.ac.jp

Tamotsu Oomori is with the University of the Ryukyus, Dept. of Chemistry, 1 Senbaru, Nishihara-cho, Okinawa, Japan (e-mail: oomori@sci.u-ryukyu.ac.jp).

adding proteins in the solution. The amount of protein *concentration* in the organic matrix solution was measured by the method of Lowry et al., [17].

C. Atomic Force Microscope (AFM)

The samples for AFM were obtained according to the method of *in vitro* experiments as described elsewhere [1] The atomic force microscope (AFM) maps the topography of surface. In phase imaging, a variant of tapping mode, the phase lag of the cantilever oscillation relative to the signal sent to the cantilver's piezo driver is used as a basis for image generation. Phase images can be generated as a consequence of variations in material properties such as friction, AFM observations were conducted with SPM-9500, SHIMADZU, at room temperature and air.

III. Results and Discussion

A. Colony and sclerites

Two techniques (mechanical and chemical) were used to separate the sclerites from the colony. Mechanically and chemically treated purified sclerites and their location in the colony have been presented in Fig. 1. A young colony of L. crassum and an apical part of the thumb-shaped lobe of the colony have been shown in Fig. 1a and 1b, respectively. Fig. 1c showed a computer model of tentacle that contains endoskeletal sclerites. Two types of sclerites, rod-like and dumbbell-shaped were observed under SEM (Fig. 1d). As shown in Fig. 1d, upon using bleaching (1% NaClO, 2h), there was no contamination and sclerites were isolated intake from the soft tissues without any damaging.

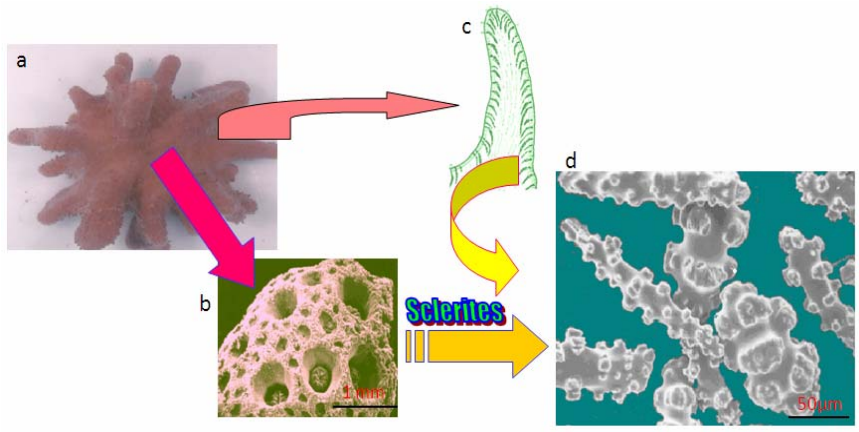

Fig. 1. Separation of sclerites (endoskeletons) from the colony of soft coral. (a)Top view of a young colony of *Lobophytum crassum (b)* Scanning electron micrographs (SEM) of an apical part of the thumb-shaped lobe of the colony. (c) A computer model of tentacle, as shown in containing sclerites. (d) Two types of sclerites, rod-like and dumbel-shaped were observed under SEM.

Soft corals growth and calcification are completely controlled by sclerites [1] and they are intensively used by scientists to assist in the identification of the soft coral species. The spicules are usually found in the axis as well as the cortex of the colonial skeleton, although they may also be present in the tentacles, pharynx and upper part of the autozoids [18]. As the results indicate, the sclerites in *L.crassum* are quite different from that of the Gorgoniacea described by Schmidt [19]. He distinguished four different spicule types. Scale-shaped or rounded spicules with a spherulitic structure are formed by some gorgonians. Spindles to rod-shaped sclerites with calcite c axes aligned with the sclerite long axis are common among gorgonians and pennatulids. Sclerite shapes are species determined and within a species they generally differ according to the anatomical site at which they are formed.

B. In vitro crystallization

Effect of cell-free proteins in the nucleation and precipitation of $CaCO_3$ crystals were studied by recording the decline of pH resulting from the reaction: $Ca^{2+} + HCO_3^- \rightarrow CaCO_3 + H^+$. Recording of a series of precipitation experiments are shown in Fig. 2. The rate of $CaCO_3$ precipitation was determined by the addition of the matrix proteins of sclerites from *L. crassum.*

Three mL of 20 mM $CaCl_2$ (pH approximately 8.7) was added to a reaction vessel containing 20 mM $NaHCO_3$ (pH approximately 8.7). As shown in Figure 2A, when H_2O was added to solutions at the start of precipitation, pH was decreased quickly. However, when the organic matrix protein (containing 11µg) was added to solutions during the time course of precipitation, the pH decrease stopped immediately, suggesting inhibition of nucleation. Subsequently when one-tenth of the amount of organic matrix protein (containing 1.1 µg) was added to solutions at the same time, there is only a slight pH decrease. In one experiment, when the organic matrix proteins (containing 11 µg) was added to solutions after 2 minutes in which precipitation had already begun, further precipitation was completely inhibited (Fig.2B). In order to compare the efficacy of this study, we applied commercial calmodulin protein during experiments. As shown in Fig. 2C, when H_2O was added to solutions at the start of precipitation, pH was decreased quickly but when calmodulin (containing 25 µg) was added to solutions, the pH decrease stopped immediately. Again, one-tenth of the amount of calmodulin (containing 2.5 µg) was added to solutions at the same time, there is only a slight pH decrease. When phosphovitin (Sigma), a phosphoprotein that inhibits calcium phosphate crystal growth [20], was tested in this system (Fig. 2D). Upon addition of phosvitin, we observed only slight inhibition of the rate and negligible inhibition of precipitation, and then only with quantities of phosvitin greater than the

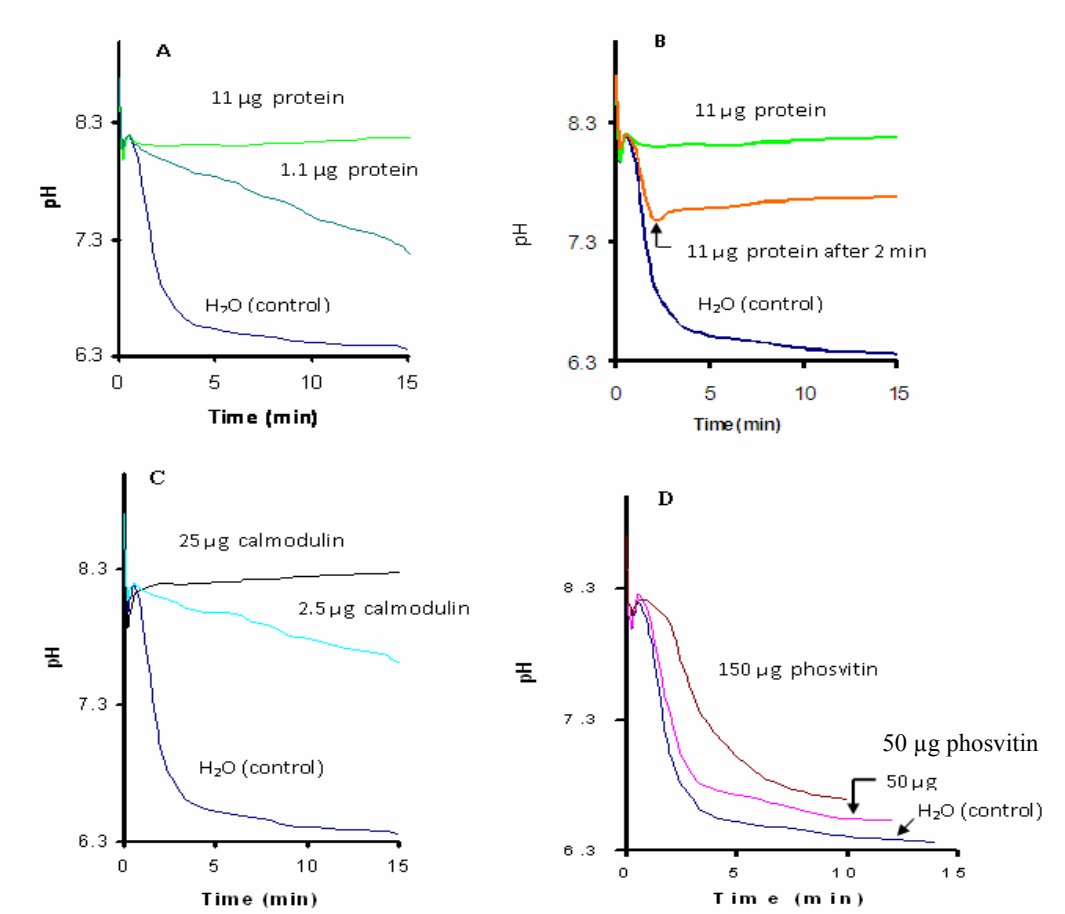

Fig 2. Recording of $CaCO_3$ precipitation by the addition of cell-free organic matrix proteins of sclerites from *L. crassum*. Three ml of 20mM $Cacl_2$ (pH approximately 8.7) was added to a reaction vessel containing 20mM $NaHCO_3$ (pH approximately 8.7). (A) Effect of proteins on precipitation. (B) Effect of protein addition after onset of $CaCO_3$ formation (arrow). (C) Effect of calmodulin on $CaCO_3$ precipitation. (D) Effect of phosvitin on $CaCO_3$ precipitation

quantities of matrix normally employed in these experiments. We didn't find any effect of phosphoprotein in this system.

Upon mixing of $CaCl_2$ and $NaHCO_3$ the pH drops rapidly and then remains stable until nucleation occurred, and it again declined as visible precipitate formed. Subsequently, a precipitate is formed causing a further decrease of pH (Fig.2A), because an addition of H_2O had no effect on precipitation. Thus, precipitation proceeds by the following steps. First, crystal precursors are formed (probably amorphous $CaCO_3$ hydrates, cf. Nancollas), followed by crystal growth resulting in a visible precipitate. Generally, a precipitate is formed within five minutes. Upon addition of organic matrix protein (containing 11µg) is added to solutions at the start of precipitation, the decrease of pH stops immediately; but when protein (containing 1.1 µg) was added to solutions at the same time, the pH value decreased slightly. The cause of the decrease in pH values was probably due to the low volume of protein. These results can be explained by assuming an interaction of the matrix protein with crystal nuclei, thus blocking further growth of the latter. These results also demonstrate that the organic matrix protein has the capacity to regulate both nucleation and crystal growth. The addition of protein (containing 11µg) to solutions 2 minutes after the onset of precipitation, stops the decrease of pH completely (arrow in Fig. 2B), probably through binding to crystal nuclei and to the growth sites of the crystals. The results reported here directly demonstrate regulation of crystal nucleation and growth by a protein in a biocalcification system.

C. Micro- and nanostructures of $CaCO_3$ crystals

The surfaces of the seed crystals (104 calcites) and their growth steps influenced by cell-free proteins were observed with AFM. As shown in Fig. 3, no growth steps were observed on the surfaces of crystals without any protein induced (Fig. 2A, B). Since the Mg^{2+}-ion is influential to make aragonite crystals in the sea environment; we examined

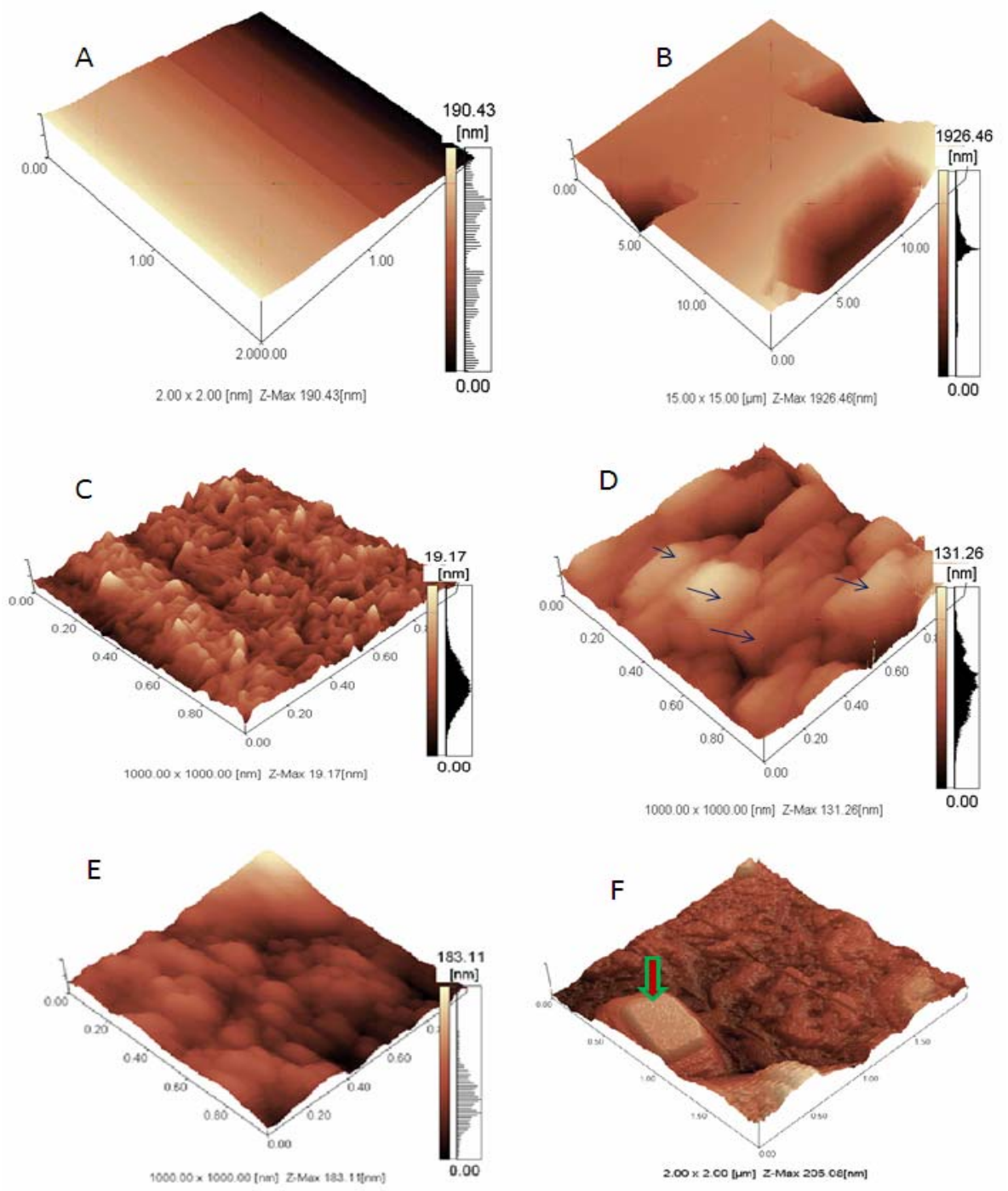

Fig. 3 Observation of the transition from aragonite to calcite growth as induced by cell-free proteins. AFM of (A) blank crystal seed, (B) crystallization on the seed crystal in the supersaturated solution of without protein and without Mg^{2+}. (C) AFM of crystallization on the seed crystal in the supersaturated solution of without protein and with Mg^{2+} (50mM), indicates needle-like aragonite formation. (D) AFM of crystallization on the seed crystal in the supersaturated solution with protein (containing 0.5µg/mL) and with Mg^{2+} (50mM), showing the influence of proteins in the morphology change of crystal (arrows). (E, F) AFM of crystallization on the seed crystal in the supersaturated solution with protein (containing 1.4µg/mL) and with Mg^{2+} (50mM), showing rhombohedral calcite crystals growth steps in which indicates perfect proteins concentration of the transition from aragonite to calcite growth during biocalcification process. The *arrow* in Fig (F) indicates a rhombohedral calcite crystal.

the crystallization at the same experimental time as we performed in our laboratory with induced proteins. When we added 50mM Mg^{2+}-ion in the experimental design, we observed the step edges are nanostructures aragonites (Fig. 3C). Subsequently, when 0.5µg/mL concentration of proteins were added to solutions at the same experiment and same experimental time, the step edges were changed (Fig. 3D) and calcite growth steps were started (arrows). In one experiment, when the proteins (containing 1.4µg/mL) was added to solutions at the same experiment and same experimental time, the step edges of calcites in nanometer scale were clearly visible (Fig. 3E, F). The *arrow* in Fig. 3F shows a microstructure (approximately 0.5 µm) of calcite crystal obtained by the influence of cell free proteins. Several experiments in the biocalcification process (data not shown) were conducted in the presence of different concentration of proteins to obtain complete transition from aragonites to calcites, and finally we found the protein concentration of 1.4µg/mL is suitable.

Previously, we reported that the matrix proteins in soft corals are aspartic acid-rich [8-10] and two proteins of *L. crassum* MPL-1 and MPL-2 showed Ca^{2+}-binding and significantly carbonic anhydrase (CA) enzyme activities [21]. It is reported that aspartic acid-rich proteins [8-12] and CA enzyme are potentially involved in the process of biocalcification [21]. However, transition from aragonite to calcite growth as induced by cell-free proteins seem to be occurred by the influence of aspartic acid-rich and CA enzyme of which also contain Ca^{2+}-binding protein. These observations support the earlier suggestions [8] that the nucleation and growth of calcite crystals were observed by the induced of soluble and insoluble proteins in different soft coral. In conclusion, cell-free proteins in soft corals have the potential to regulate $CaCO_3$ deposition and their growth via calcified endoskeletons (sclerites). The proposed mechanisms may have direct implication for all calcified marine organisms and microbial communities, as well as in all kinds of materials synthesis

.

ACKNOWLEDGMENT

This work was supported by the Postdoctoral Research Grant of Japan Society for the Promotion of Science (JSPS)

REFERENCES

[1] M. A. Rahman, T. Oomori, Structure**,** Crystallization and Mineral Composition of Sclerites in the Alcyonarian Coral, *J. cryst. Growth*, 310:3528-3534, 2008.

[2] L. Addadi, and S**.,** Weiner, Interactions between Acidic Proteins and Crystals: Stereochemical Requirements in Biomineralization. *Proc. Natl. Acad. Sci. USA* 82, 4110-4114, 1985.

[3] S., Weiner, W. Traub, and H.A. Lowenstam, 1983. In: *Biomineralization and Biological Metal Accumulation*, eds Westbroek, P. & de Jong, EW (Reidel, Dordrecht, Holland), pp 205-224.

[4] M.J., Glimcher, 1981**.** In *The Chemistry and Biology of Mineralized Connective Tissues,* ed Veis. A (Elsevier, New York), pp 617-673.

[5] A. Veis, and B. Sabsay, 1983. In: *Biomineralization and Biological Metal Accumulation,* eds Westbroek, P & de Jong, EW (Reidel, Dordrecht, Holland), pp 273-284.

[6] S. Weiner, Organization of organic matrix components in mineralized tissues. *Amer. Zool*. 24, 945–951, 1984.

[7] M.A. Rahman, and Y. Isa, Characterization of proteins from the matrix of spicules from the alcyonarian, Lobophytum *crassum*. *J. Exp. Mar. Biol. Ecol.* 321, 71-82, 2005.

[8] M. A. Rahman, T. Oomori, *In vitro* regulation of $CaCO_3$ crystal growth by the highly acidic proteins of calcitic sclerites in soft coral, *Sinularia polydactyla,* Conn. Tissue Res. (in press) DOI:10.1080/03008200802714933

[9] M.A. Rahman, Y. Isa, A. Takemura**,** T. Uehara, "Analysis of proteinaceous components of the organic matrix of endoskeletal sclerites from the alcyonarian, *Lobophytum crassum, Calcif. Tissue Int.,* 78: 178–185, 2006.

[10] M. A. Rahman, T. Oomori, Aspartic Acid-rich Proteins in Insoluble Organic Matrix Play a Key Role in the Growth of Calcitic Sclerites in Alcyonarian Coral, *Chin J Biotech.* 24: 2127−2128, 2008.

[11] M. A. Crenshaw, *Biomineralization* 1972, p. 6.

[12] S. Weiner, Aspartic acid-rich proteins**:** major components of the soluble organic matrix of mollusk shells, Calcif Tissue Int 29 (1979) 163-167.

[13] M. A. Crenshaw and H. Ristedt, in *The Mechanisms of Mineralization in the Invertebrates and Plants,* N. Watabe and K.M. Wilbur, Eds, (University of South Carolina Press, Columbia, 1976, p. 355.

[14] M.A. Rahman, Y. Isa, T. Uehara, "Studies on two Closely Related Species of Octocorallians: Biochemical and Molecular Characteristics of the Organic Matrices of Endoskeletal Sclerites," *Mar. Biotechnol*, 8: 415-424, 2006.

[15] M.A. Rahman, Y. Isa, T. Uehara, Proteins of calcified endoskeleton: II. Partial amino acid sequences of endoskeletal proteins and the characterization of proteinaceous organic matrix of spicules from the alcyonarian*, Synularia polydactyla, Proteomics* 5: 885-893, 2005.

[16] A.H. Borman, E.W. De Jong, M. Huizinga, P. Westbroek, In: Westbroek, P., de Jong, E.W. (eds.), *Biomineralization and Biological Metal Accumulation,* D Reidel publishing, Dordrecht, Boston, London 1983, p. 303.

[17] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall) Protein measurement with the Folin phenol Reagent. *J Biol Chem* 193: 265-275, 1951.

[18] H. A. Lowenstam , S. Weiner, 1989. On Biomineralization (Chapter 5: Cnidaria), pp. 74-87.

[19] W.J. Schmidt, 1924. Die Bausteine des Tierkorpers in Polarisiertem Lichte. F. Cohen Verlag, Bonn.

[20] J.D. Termine, E.D. Eanes, K.M. Conn, Calcif. Tissue Int., 31: 247, 1980

[21] M.A. Rahman, T. Oomori, T. Uehara, Carbonic anhydrase in calcified endoskeleton: Novel activity in biocalcification in alcyonarian, *Mar. Biotechnol.* 10: 31-38, 2008.