The Role of Carbonic Anhydrase Enzyme in the Biocalcification Process of Coral and its Resilience to Global Climate Change

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Abstract-Here we report that the organic substances that participate in calcification in coral skeletons contain a carbonic anhydrase (CA) enzyme which is a biological catalyst responsible for the interconversion of CO_2 and bicarbonate. The effect of CA on the hydration of CO_2, and its precipitation in the form of calcium carbonate, was studied. The precipitation of calcium carbonate was promoted in the presence of the enzyme. However, we find that the CA acts as "keys" to control internal physiological condition of the body of corals via calcification and to enable a response to external environmental change. Therefore, the CA enzyme is considered to be key biomolecule to understand calcification mechanisms and global climate change.

I. INTRODUCTION

The present world is facing a great problem due to climate change, presumably caused by the increased input of anthropogenically produced CO_2; therefore, it is essential to conduct research to find ways to contribute to solving this problematic issue. The response of calcifying marine organisms, especially from coral reefs – arguably among the most biologically diverse and ecologically important ecosystems on the planet – could have a potential mitigating role in buffering atmospheric CO_2.

Reef building organisms make calcium carbonate skeletons and construct huge reef framework structures, which offer various kinds of space and habitats for diverse marine organisms. Biologically formed calcium carbonates are mainly calcite, aragonite and vaterite as well as high Mg-calcites. Recently, biologically formed amorphous and hydrous calcium carbonates have also been reported. During the work on biocalcification, it is seen that morphology, mineralogy and chemistry of biologically formed calcium carbonate are largely dependent on both biological species and physical-chemical environmental conditions. Proteins and enzymes may act as "keys" to control internal conditions and respond to external environmental change. Identification and elucidation of new proteins and their enzyme activity involved in calcium carbonate skeleton or spicules in corals are very important to understand the calcification process. Understanding these new proteins is also related to understanding the biodiversity of coral reef ecosystems and carbon cycling, along with global environmental change. Increasing atmospheric carbon dioxide concentrations are reducing ocean pH and carbonate ion concentrations, and thus the level of calcium carbonate saturation. Experimental evidence suggests that if these trends continue, key marine organisms, such as corals, will have difficulty maintaining their external calcium carbonate skeletons. Atmospheric CO_2 is expected to reach double the preindustrial levels by the year 2065. It is believed that the increase in CO_2 concentration is responsible for global warming. Hence, it is essential to find ways of reducing CO_2 emissions. CA enzyme could be a potential biomolecule for solving this problem.

Biological sequestration of carbon dioxide (CO_2) in geological formations is one of the proposed methods to reduce the carbon dioxide released into the atmosphere. The best way for CO_2 disposal is sequestration based on the chemical fixation of CO_2 in the form of carbonate minerals such as calcite, magnesite, and dolomite. This is a safe and permanent method of disposing of CO_2. These carbonate minerals are abundant in nature and are environmentally benign and stable. Mineralization of CO_2 can be achieved by direct contact of gaseous CO_2 with mineral sources of calcium or magnesium or by dissolving CO_2 in water and then bringing the solution into contact with the minerals. Either way will produce calcium or magnesium carbonate, which are solids and will precipitate [1, 2]. In the present work, the feasibility of using this enzyme as a catalyst for hydration of CO_2, as well as its precipitation in the form of calcium carbonate, was studied. The effects of enzyme concentration and temperature on the hydration of CO_2 and formation of calcium carbonate were investigated. The information gained from this study would be applicable to coral communities around the world in understanding the calcification mechanisms of corals along with their response to global climate change.

II. MATERIALS AND METHODS

A. Sample preparation

Sclerites were separated from the coral colony (*Lobophytum crassum* Marenzeller) according to the mechanical and chemical treatments followed by Rahman et al., 2006 [3]. Briefly, the collected sclerites were stirred vigorously in 1M NaOH for 2 hours

and subsequently in 1% NaClO solution for 2 hours 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) to remove unwanted substances.

B. CA assay

To examine the CA activity, the chemically treated sclerites were decalcified in 0.5 M EDTA (pH 7.8) overnight, followed by dialysis against H_2O for 48 hours. Proteins were separated from the soluble organic matrix of sclerites using SDS-polyacrylamide gel electrophoresis according to the method of Laemmli [4]. After electrophoresis, the bands of interest were excised from the SDS-PAGE and the pure proteins were extracted by electro-elution treatment, using the Electro-eluter (Model 422, Bio-Rad) according to the procedure of Rahman et al., [5]. The Micron centrifugal Filter Devices (Millipore) were used for further purification of the sample.

The amount of protein concentration in each fraction was measured by the method of Lowry et al. [6] using chicken ovalbumin (Kanto chemical) as a standard protein followed by a spectrophotometric assay of protein. We measured CA activity using the CO_2-Veronal indicator method [7] as follows. Six drops of phenol red (200 µl), 3 ml of 20mM Veronal buffer (Sodium 5, 5-Diethylbarbiturate, pH 8.3), 0 .5 ml of a sample were mixed and placed in ice water; then the reaction was started by adding 2 ml of ice-cold water saturated with CO_2 followed by observation of the time until the color changed from red to yellow for a pH drop to 7.3. The EU was calculated according to the following equation.

$$\text{Activity unit (EU)} = (\text{To} - \text{T})/\text{T}$$

[where T and To are the reaction times required for the pH change from 8.3 to 7.3 at 0°C with and without a catalyst, respectively].

C. Enzymatic Hydration of CO_2. Carbonic anhydrase catalyzes the hydration reaction of CO_2, and consequently, hydrogen ions are transferred between the active site of the enzyme. This results in a change in pH. Therefore, measuring pH via the delta pH method is a viable method to monitor the progress of this enzymatic reaction [8]

C. Enzymatic Precipitation of Calcium Carbonate.

The influence of CA enzyme on the precipitation of CO_2 in the form of calcium carbonate ($CaCO_3$) was studied according to Mirjafari et al [9] and Rahman et al. [10]

III. RESULTS AND DISCUSSION

A. CA activity

Carbonic anhydrase (Tashian, 1989, EC 4.2.1.1) [11] is a Zinc-containing enzyme which catalyzes the reversible conversion of CO_2 to HCO_3 (equation 1 below), which would then be available for $CaCO_3$ formation (equation 2).

$$CO_2 + H_2O \leftrightharpoons HCO_3^- + H^+ \text{---------(1)}$$

$$Ca^{2+} + HCO_3 \leftrightharpoons CaCO_3 + H^+ \text{------(2)}$$

For understanding biocalcification process in corals and their response to climate change, we purified proteins and enzyme from soft coral sclerites, *L. crassum* as a model. In the present work, we purified a protein with a molecular mass of about 67-kDa (MPL-2), which has both calcium-binding and CA activities (Fig. 1, lane 2). Information for calcium-binding protein has already been reported [3]**.** We excised the protein bands from the SDS-PAGE and extracted the purified proteins by electro-elution treatment. The SDS-PAGE analysis of the preparation showed four proteins with the apparent molecular masses of 102, 67, 48 and 37 kDa (Fig. 1, lane 1) that we named MPL-1, MPL-2, MPL-3 and MPL-4 respectively. We confirmed CA protein (MPL-2) by further SDS-PAGE analysis (Fig. 1, lane 2).

In order to identify CA activity associated with the purified proteins, the samples were assayed independently. Fig. 2 shows that the MPL-1 and MPL-2 possessed specific CA activity; where MPL-2 showed higher activity. The CA activity of both the matrix protein (MPL-2) and bovine erythrocyte enzyme (BECA) was inactivated by heat treatment at 100°C for 10 min, which showed no activity. Since MPL-2 showed the highest and most significant activity among the four proteins, we can consider only MPL-2 to be a CA domain protein in *L. crassum.*

To our knowledge, MPL-2 described in this study is the third identification of biomineral matrix proteins which has CA activity, following to pearl oyster shell proteins, nacrein and N66. In addition, MPL-2 is the first-

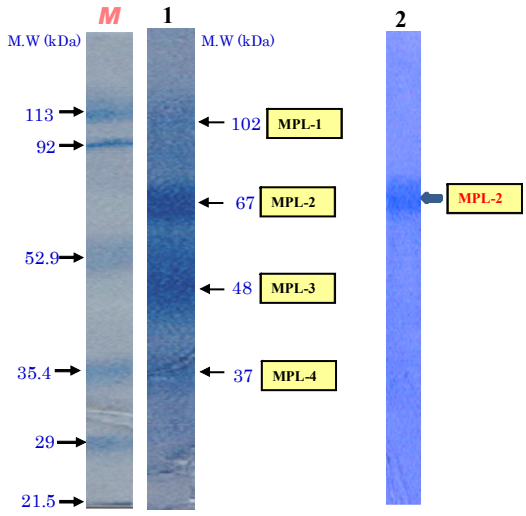

Fig. 1. Identification of proteins. Lane 1, SDS-PAGE separation of the total assemblage of soluble matrix proteins.with CBB staining. Lane 2, Identification of single protein by electroelution treatments. M, Protein marker. *Arrows* indicate significant protein bands. *Arrow* in lane 2 indicates significant CA domain protein in *L. crassum.*

reported protein that has activity both for calcium binding and CA. It is unknown whether nacrein (reported by Miyamoto et al., 1996) [12] is a calcium binding protein because stains-all staining cannot evaluate calcium binding activity. Thus CA domain protein MPL-2 is highly important to discuss coral biomineralization and their response to global climate change. However, internal and external reactions of coral body including hard endoskeleton of sclerites are occurred with biocalcification process. In the mineralization of CO_2, calcium carbonate is produced through a reaction between calcium ions and aqueous CO_2. The following reactions take place in this process:

(1) First, gaseous CO_2 dissolves in water to form aqueous CO_2 (reaction 1).

$$CO_{2(g)} \leftrightarrow O_{2(aq)} \qquad (1)$$

(2) Then, aqueous CO_2 reacts with water to form carbonic acid:

$$CO_{2(aq)} + H_2O \xrightleftharpoons[k_{-2}]{k_2} H_2CO_3 \qquad (2)$$

$$k_2 = 6.2 \times 10^{-2}\ s^{-1} \quad \text{and} \quad k_{-2} = 23.7\ s^{-1}$$

$$K_2 = \frac{k_2}{k_{-2}} = \frac{6.2 \times 10^{-2}}{23.7} = 2.6 \times 10^{-3}$$

(3) In the next step, carbonic acid dissociates to bicarbonate and carbonate ions:

$$H_2CO_3 \xrightleftharpoons[k_{-3}]{k_3} H^+ + HCO_3^- \qquad (3)$$

$$HCO_3^- \xrightleftharpoons[k_{-4}]{k_4} H^+ + CO_3^{2-} \qquad (4)$$

The equilibrium constant of reaction 3 is equal to 1.7×10^{-4}:

$$K_3 = \frac{k_3}{k_{-3}} = \frac{8 \times 10^6\ (s^{-1})}{4.7 \times 10^{10}\ (s^{-1})} = 1.7 \times 10^{-4}$$

Reaction 3 is very rapid and is virtually diffusion controlled.

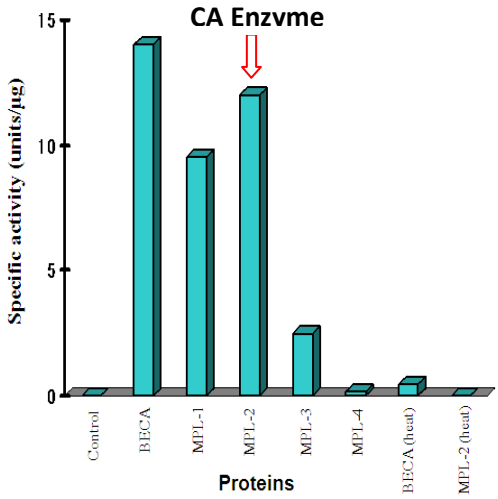

Fig. 2 Determination of CA activity with purified matrix proteins. Specific activity was measured in the presence of matrix proteins at concentration of 1.5 ml (contain 30μg protein) for each protein; MPL-2 showed the highest activity. H_2O (control) and bovine erythrocyte CA (BECA) was used as a standard in comparing the efficacy of CA proteins. The inset shows the mean values of CA activity for four matrix proteins. Ten experiments were conducted for each protein. The novel matrix protein of MPL-2 showed significant CA activity.

(4) At the end, in the presence of calcium cations, calcium carbonate forms and precipitates:

$$Ca^{2+} + CO_3^{2-} \rightarrow CaCO_3\downarrow \qquad (5)$$

Among reactions 1−5, reaction 2 is the slowest, and it is the rate-limiting step. It is proposed that a biological catalyst be used to increase the rate of this reaction.[13-15]. The biological catalyst for this reaction is enzyme carbonic anhydrase.

B. Enzymatic Hydration of CO_2

As shown in Figure 3, addition of CO_2 to the reaction mixture at time zero tended to drop the pH of the mixture to the low values, but the presence of the buffer balanced this effect, and the pH leveled off at a value slightly lower than the pH of the buffered solution.

Since the initial rate of pH drop (r_A) is equal to the slope of the curves at the first few seconds of the reaction, to compare the rate of pH drop at different concentrations, the slope of the curves in Figure 3 were calculated for the early part of the reaction. It is clearly observed in this figure that the pH drop depends on the concentration of the enzyme, as shown in Figure 4. At higher enzyme concentrations, pH drops more quickly. Also, the results show that in the absence of the enzyme (control run) the rate of pH decrease is much slower compared to the rate of pH drop in the presence of the enzyme, even at low concentration of the enzyme (e.g., 0.2 μM). These results demonstrate that enzyme carbonic anhydrase increases the rate of hydration of CO_2, even at low concentrations.

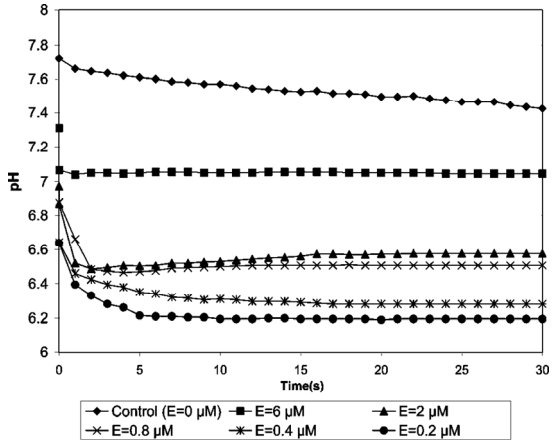

Fig. 3. pH experiments at $T = 0$ °C and in the presence of buffer.

C. Enzymatic Precipitation of Calcium Carbonate

The results of precipitation experiments showed that the enzymatic precipitation of $CaCO_3$ was not dependent on the concentration of CA enzyme. With CA enzyme at concentrations of 3 and 6 μM, the weight of $CaCO_3$ was 0.2098 and 0.2106 g, respectively, at 0 °C and 0.1280 and 0.1283 g, respectively, at 30 °C. These numbers are very close, and the difference is negligible. These results are summarized in Table 1.

The amount of $CaCO_3$ decreased as the temperature increased. The weight of $CaCO_3$ at 0, 30, and 50 °C was 0.2098, 0.1283, and 0.096 g, respectively. This is because the solubility of CO_2 in water decreases with increasing temperature [16]. This result did not depend on the temperature or concentration of the enzyme. When the buffer is absent from the reaction mixture, addition of CO_2 to the mixture drives the pH down to low values (near 4). The chemistry of CO_2 hydration and bicarbonate dissociation shows that, in low pH, there is not enough carbonate ion present [17]. As a result, the solution does not become saturated with $CaCO_3$. This is the reason precipitation was not observed in this condition.

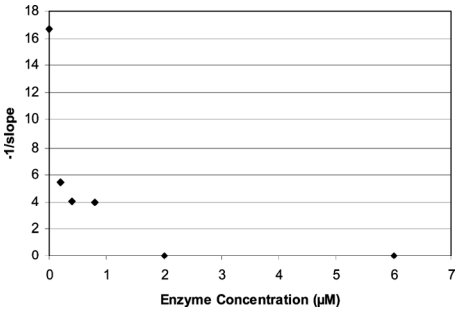

Fig. 4. Comparison of the rate of pH drop for different enzyme concentrations at 0 °C.

In the absence of the enzyme, precipitation was observed after 2 h. The amount of $CaCO_3$ was almost the same as its amount in the presence of the enzyme (Table 1). However, Figure 5 indicates that precipitation of calcium carbonate was much faster in the presence of CA enzyme. This figure presents a comparison of the precipitation in the presence and absence of the enzyme. It is clearly seen that, in the presence of the enzyme, $CaCO_3$ reached its maximum value in less than 10 min; however, when no enzyme was added to the reaction mixture, the formation of calcium carbonate took place very slowly.

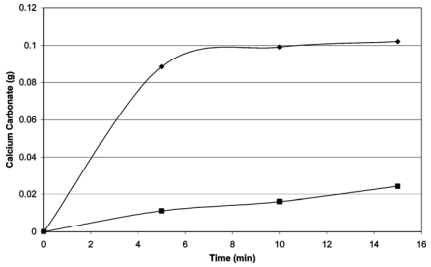

Fig. 5. Comparison of precipitation with and without enzyme.

Carbonic anhydrases are very well-known enzymes that are ubiquitous in nature. They can be found in animals and plants and even in the human erythrocyte. They exist in different forms, with different structures and molecular weights, and their activities vary from one to another.[11] They are among the fastest enzymes known. For instance, each molecule of isozyme C from the human body can catalyze 1.4×10^6 molecules of CO_2 in 1 s [18].

In the presence of an anhydrase enzyme, the mechanism of hydration of CO_2 changes completely. The evidence suggests that the catalysis of CO_2 hydration is initiated by the nucleophilic attack on the carbon atom of CO_2, by zinc-bound OH^-, to produce bicarbonate, which is then displaced from zinc by a water molecule [19]. In this study, the Mineralization of carbon dioxide is investigated as a method of converting CO_2 to mineral carbonates. The slow rate of hydration of CO_2 has been a limiting factor to make this method widely accepted. Carbonic anhydrase enzyme isolated from soft corals sclerites has been shown to be credible as biological catalysts to overcome this shortcoming. The results showed that this enzyme was a very effective catalyst. It promoted the hydration of CO_2 and, consequently, the precipitation of $CaCO_3$.

TABLE 1.
Summary of $CaCO_3$ Precipitations

Set	temp (°C)	enzyme concn (μM)	wt of precipitate (g)	no. of runs	error (%)
1	0	3	0.2198	2	0.1
2	0	6	0.2106	4	4.2
3	0	6	0	3	N/A
4	0	no enzyme	0.19	2	1
5	30	3	0.1280	2	0.16
6	30	6	0.1283	4	1.8
7	50	6	0.096	3	1.7
8	50	no enzyme	0.0941	2	1.5

ACKNOWLEDGMENT

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