BIOCALCIFICATION AND ACTIVITY OF CARBONIC ANHYDRASE IN THE EXTRACELLULAR MATRIX OF CORAL

THESIS

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ABSTRACT

Crushed exoskeletons of the coral, *Astrangia astrangia*, were treated with sodium hypochlorite (1.5%), SDS (0.5%) and then decalcified with EDTA. Upon purification by affinity chromatography extracellular proteins with Mr of 31, 53, and 63 kDa were distinguished using SDS/PAGE gel. The proteins appear to be extracellular isozymes of carbonic anhydrase (CA). Bovine serum CA II antibodies cross-reacted with the proteins isolated via affinity chromatography (ELISA and Western Blots). High CA enzyme activity and the inhibition of CA with acetazolamide, all support the contention that an extracellular CA is present in the exoskeleton. Cleaning the crushed exoskeleton with both SDS and sodium hypochlorite to remove cellular proteins that are not entombed in the calcite prior to CA extraction we assert that CA is secreted extracellularly along with the matrix proteins. The total water-soluble proteins (WSP) in the exoskeleton of the coral, *A. astrangia*, was 14 ± 7 mg – protein/100g exoskeleton whereas CA isozymes represent 224 ± 108 μg – protein/100g exoskeleton (N=6) or 1.71% the total WSP. These data illustrate that coral extracellular CA isozymes do function in exoskeleton mineralization by maintaining the bicarbonate concentrations at equilibrium so as to maintain the availability of carbonate ions used in mineralization.

Introduction

Many studies on biomineralization of hard and soft corals have been conducted over the last few years with the accumulation of a considerable amount of information (Goreau, 1961; Clausen and Roth, 1975; Tambutte, 1996), but the actual details of the calcification mechanism still remains an enigma. Most of the studies focused on symbiotic relationships of corals with zooanthellae, growth rates, ecology, and calcium transport. Many of the studies also implicate a role for carbonic anhydrase (CA) in biomineralization. Goreau (1961) used radioactive isotopes to examine growth and calcium deposition in the formation of coral reefs. Although the actual mechanism of calcification was not identified, he believed that it was localized just outside the calcioblastic epidermis. Here calcium is absorbed on a mucopolysaccharide-like material, which forms part of the organic matrix. The matrix acts as a template upon which the initial stages of skeleton mineralization occurs (Goreau, 1961).

Following the studies by Goreau (1961), Clausen and Roth (1975) examined the effects of environmental factors on coral calcification with the use of radioactive Ca^{45}. In measuring growth rates of two hermatypic corals, *Pocillopora damicornis* and *Porites compressa*, the terminal end of branches were observed to calcify faster than lateral sides in both corals. Using a compartmental approach, Tambutte et al. (1996) used radioactive isotopes to characterize calcium compartments within coral colonies and the movement of calcium between them. Their study demonstrated that calcium for skeletal growth in corals must pass through calcium channels that are responsible for passive entry into the cells (Tambutte et al., 1996). Although their findings contradict earlier studies by

Johnston (1980) that calcium is transported via a paracellular/intercellular route, they observed that calcium travels via a transcellular route.

The structure of coral exoskeletons differs in many aspects among the hundreds of different species (Chave, 1971). Skeletons maybe composed of individual spicules, spicule aggregates (soft corals), or calcareous masses (hard corals), that are formed by intracellular or extracellular calcification (Allemand and Grillo, 1992). In some unknown manner the calcification mechanisms seem to be associated with CA (E.C. 4.2.1.1.). Carbonic anhydrase is a key enzyme in the hydration of CO_2 and the formation of bicarbonate ions that are used in mineralization of coral exoskeletons (Lucas and Knapp, 1996). To maximize the formation of carbonate ion via the uncatalyzed reaction (UC), a saturated solution of bicarbonate is needed. Carbonic anhydrase catalyzes and maintains the following equilibrium:

$$CO_2 + H_2O \overset{CA}{\leftrightarrow} H_2CO_3 \overset{CA}{\leftrightarrow} HCO_3^- + H^+ \overset{UC}{\leftrightarrow} CO_3^- + H^+$$

At physiological pHs (7-8) the reaction is favored towards bicarbonate (pH=7.4; 1:6500); (Voet et al., 1999). Formation of carbonate ion via bicarbonate is the only source of carbonate used in calcareous exoskeletons. Bicarbonate is supplied by both cell metabolism and the oceanic environment. Because the bicarbonate concentration in the ocean is 2.34 mM seawater, it is the source of most of the bicarbonate ions (Barnes, 1954). Although the reaction from bicarbonate to carbonate is highly favored in the direction of bicarbonate ions, maintenance of saturation levels of bicarbonate via action of CA would facilitate the formation of calcium carbonate because of the enhanced availability of carbonate ion. High concentrations of bicarbonate ions raise the quantity of carbonate ions proportionally.

The calcium content of the ocean is high ([Ca] = 10.23 mM seawater; Barnes, 1954) and is not assumed to be limiting. The general consensus is that the source of both ions is from seawater. The coral exoskeleton is composed mostly of aragonite, which allows for some $SrCO_3$ to form. The quantity of Sr in aragonite is dependent on the availability in the ocean ([Sr]=0.156 mM seawater). The Sr content of aragonite can increase with the increase in seawater temperature (Mitsuguchi et al., 1996).

Although CA of mammals has been extensively studied (Pullan and Noltmann, 1984; Deutsch, 1984), the CAs of only a relatively few species of invertebrates and lower vertebrates have been examined and even then, not in detail. Little is known about the biochemical role of CA and its relationship to the formation and degradation of calcified hard tissues, other than it is someway related (Lucas and Knapp, 1996; Simkiss and Wilbur, 1989). Since CA is involved in coral skeletogenesis, it is assumed that the skeletal forming epithelial tissue would have high activity of the enzyme (Isa and Yamazato, 1984).

A most striking feature of the stony coral, *Astrangia astrangia*, is its ability to form a massive calcareous exoskeleton of aragonite that forms a supporting base upon which the polyps live (Goreau, 1961). Before initiating formation of the exoskeleton the larva must first attach by the aboral surface; then the ectoderm at the site of attachment undergoes a process of cell flattening (Vandermeulen, 1974,1975; Johnson, 1978). After metamorphosis of the larva into a polyp these cells will become the squamous calcicoblastic ectoderm that forms the calcareous skeleton (Simkiss and Wilbur, 1989). The rest of the ectoderm then secrets mucus and forms an epithelium over the whole organism (Simkiss and Wilbur, 1989). The calcioblastic epithelium continuously spreads

out forming a basal plate and allows the metamorphosing larva to attach, and then develop into a polyp. The edges of the basal plate extend as a crystalline sheet, called the epitheca. The epitheca may be the site at which calcium carbonate deposition takes place in order to increase thickening (Barnes, 1972). The organic matrix of the skeleton is released by the calicoblastic epithelial cells into the subepithelial space where crystals form and grow within the matrix. It is this matrix that directs crystal formation (Falini et al., 1996; Belcher et al., 1999).

The organization of the coral is characterized by two epithelial layers, an outer ectoderm and an inner endoderm (Simkiss and Wilbur, 1989; Kozloff, 1990; Moore, 2001). These two epithelia are separated by the jelly-like mesoglea which remains flexible for support and contains some cells and connective tissue fibers but is not itself a cell layer (Moore, 2001). The mesoglea is strengthened by the deposition of intracellularly formed spicules rather than by extracellular mineralization (Simkiss and Wilbur, 1989).

Symbiotic zooanthellae found in the endoderm provide the coral with some nutrients and assist in the uptake of nitrates and phosphates; however, their chief importance seems to be that photosynthesis facilitates calcification by removing carbon dioxide ($2HCO_3^- \Rightarrow CO_3^- + H_2CO_3 \Rightarrow H_2O + CO_2$ (Moore, 2001; Benesch, 1984). Removal of CO_2 shifts the reaction towards removal of carbonic acid following carbonate formation. In light, zooanthellae fix CO_2, thus removing carbonic acid and increasing the rate of calcification; whereas in the dark, removal of carbonic acid depends on diffusion into the ocean where the acidity is neutralized (Benesch, 1984). Since the zooanthellae are always in the endodermal layer which is some distance from both the calcifying cells

of the epidermis and the fastest growing regions of coral at the branch tips (often devoid of zooanthellae), it has been suggested that their influence is general and not at the specific site of mineralization (Simkiss and Wilbur, 1989). Removal of hydrogen ion via photosynthesis ($H_2CO_3 \Rightarrow CO_2 + H_2O$) would definitely assist in mineralization. The active transport of ions by ATP for rapid movement through the ectoderm and then diffusion through the mesoglea to reach the endoderm for calcification is important also for secretion of the matrix and cell stability (Simkiss and Wilbur, 1989). The loss of ATP would slow down the rate of calcification due to the lack of ions being transported through the matrix for calcification by the calicoblastic cells. Calcium is actively absorbed from seawater and combines with bicarbonate to form calcium carbonate, which makes up the primary deposition of corals. The bicarbonate is formed from CO_2 via carbonic anhydrase (Goreau, 1961). The calcification can be inhibited by acetazolamide and other known CA inhibitors. Acetazolamide does not completely stop the calcification but greatly reduces the effects of CA and reduces the availability of bicarbonate ions for calcification (Prosser, 1973).

The focus of this paper centers on (1) demonstrating that CA is secreted along with the extracellular matrix and on (2) showing that the enzyme may play a role in the maintenance of high bicarbonate ion concentrations in the exoskeleton matrix where it facilitates the formation of calcium carbonate. Rapid formation of the calcium carbonate would allow for the continuous growth of polyps stemming from the calcioblastic ectoderm of the coral. A detailed analysis of CA in the formation of exoskeletal and polyp components offers an opportunity to establish the physiological role of this family of enzymes in the growth of corals. These data might also expand our knowledge of the

molecular evolution of CA, and the ecological impact of CA on the distribution and occurrence of organisms with highly calcified shells (Lucas and Knapp, 1996).

The specific objectives of this research on coral CA were: (1) to isolate carbonic anhydrase from the extracellular matrix of coral, (2) show that the extracellular carbonic anhydrase is active and, (3) to show the influence of CA inhibitor acetazolamide.

Methods and Materials

The extraction procedure of the corals' soluble proteins from their exoskeletons is similar to the procedures used by Miyamoto et al. (1996), whereas the purification procedure followed was similar to that Osborne and Tashian (1975), Chegwidden (1991), Bering et al. (1998), and Lane and Moral (2000).

Whole exoskeletons are cleaned of all visible debris, soaked 6 hours in 1.5% sodium hypochlorite, rinsed in deionized water (DI), soaked 6 hours in 0.5% sodium dodecyl sulfate (SDS), rinsed again in DI water, again soaked 6 hours in 1.5% sodium hypochlorite, dried and then ground to a powder in a Thomas-Wiley Laboratory Mill. Coral powder (100g) is then soaked 6 hours in 0.5% SDS, rinsed in DI water, soaked 1 hour in 1.5% sodium hypochlorite, rinsed in DI water and dried. The coral powder is placed in 1000 ml 0.025 M Tris buffer (pH 8.0, 0.01% sodium azide, 1mM 2-

mercaptoethanol). Then to dissolve the calcite/aragonite and remove the calcium, equal equivalents of Na_2H_2EDTA (372g/100g $CaCO_3$) are added slowly while stirring at room temperature. The CO_2 released may bubble over. The acidity of Na_2H_2EDTA (pH > 5) slowly dissolves the calcium carbonate. A 10% excess of Na_2H_2EDTA (37.2g/100g $CaCO_3$) facilitates dissolution of calcium carbonate. Upon decalcification (bubbling stops) the pH is adjusted to pH 9 with NaOH. To accommodate CaEDTA solubility DI water is added until all the EDTA is dissolved, and the stirred solution becomes a clear light brown. At alkaline pHs EDTA binds calcium much more efficiently. With continuous stirring at room temperature extraction is complete after 24-48 hours. All other extraction procedures are conducted in a cold room (4°C).

The coral extract (usually 1.51 M$CaCO_3$) is dialyzed three times against 0.025 M Tris buffer (pH 8.0, 0.01% sodium azide, 1mM 2-mercaptoethanol) in a cold room (4°C) to remove CaEDTA. Each dialysis is for at least 6 hours against 10X fresh buffer. Gentle extraction of protein from the exoskeleton via EDTA and the subsequent dialysis is a slow and tedious process requiring several days.

Following dialysis to remove CaEDTA, total proteins are then precipitated at 4°C with cold 80% saturated ammonium sulfate (34g/l) in 0.025 M Tris buffer (pH 7.0, 0.01% sodium azide, 1mM 2-mercaptoethanol), and centrifuged at 10,000xg for 20 minutes in a Sorvall RC5B refrigerated centrifuge. Ammonium sulfate precipitate is collected, dissolved in 50 ml 0.025 M Tris buffer (pH 7.0, 0.01% sodium azide, 1 mM 2-mercaptoethanol), and dialyzed three times. The first two dialyses are against 2000 ml of 0.025 M Tris buffer (pH 7.0, 0.01% sodium azide) at 4°C for at least 6 hours each. The

third dialysis also contains 0.01% $ZnCl_2$ to replace the Zn lost to EDTA. The dialysate then is centrifuged at 10,000xg to separate the water insoluble proteins (WIP) from the water soluble proteins (WSP). The supernatant (WSP) is retained, the precipitate washed with 0.025 Tris buffer (pH 7.0, 0.01% sodium azide, 1mM 2-mercaptoethanol) twice, centrifuged and combined with the initial supernatant.

The combined supernatants (WSP) are placed in a 50 ml beaker with one gram of wetted (0.025 M Tris, 0.25 M K_2SO_4, pH 7.0) affinity beads specific for $Ca^{==}$ (Bio-Gel A: carboxymethyl agarose containing a ligand of 4-aminomethylbenzenesulfonamide; BIO-RAD) and shaken for several hours prior to placing on a gravity pre-packed affinity column (25 x 1.5 cm) with 8cm of wetted affinity resin beads in place (Bering et al., 1998). Following application of the sample the column is then washed with 6 volumes of 0.2 M K_2SO_4/0.025 M Tris buffer (pH 7.0) and 4 ml fractions collected on Pharmacia LKB Fraction Collector until absorbance at 280 nm is constant. All of the WSP except the resin bound carbonic anhydrase is eluted with 4 volumes (approximately 60 ml) of 0.4 M KSCN/0.025 M Tris buffer (pH 7.0) by competitive inhibition and collected in 4 ml fractions as above. The KSCN fractions are concentrated to 200µl by using Millipore Ultrafree-4 Centrifugal filters, and washed three times with 4 ml of 0.025 M Tris buffer (pH 7.0) to remove KSCN.

Total WSP in the combined 0.2 M K_2SO_4/0.025 M Tris buffer (pH 7.0) fractions and the carbonic anhydrase in the 0.4 M KSCN/0.025 M Tris buffer (pH 7.0) elutes were estimated spectrophotometrically and the percentage of carbonic anhydrase (protein) in the total WSP fraction calculated (µg - protein/ml = $183 * A_{230\,nm} * 75.8 * A_{260\,nm}$; Kalb and Bernlohr, 1977). The carbonic anhydrase was concentrated again with Millipore

Ultrafree-4 centrifugal filter tubes to a volume of 200 µl. Aliquots of CA were used for Western Blots (Towbin et al. (1979) and Hawkes et al. (1982)), SDS/PAGE and ELISAs - enzyme immunoassays using rabbit antibodies to bovine carbonic anhydrase (Chemicon #AB1243). The reaction was followed with a rabbit/mouse rapid staining kit (Sigma Chemical #89H4879). Polyacrylamide gel electrophoresis (SDS/PAGE; 12.5% acrylamide) was used to ascertain purity and estimate the relative molecular mass (Mr) of the nacrein protein/carbonic anhydrase (Laemmli, 1970). A set of molecular weight standards (10 to 250 kD; BIO-RAD 161-0362) were run in two lanes along with concentrated sample. To locate the proteins, gels were stained with Coomassie blue, and then destained. Exoskeleton calcium was determined by atomic absorption spectrometry and reported as calcium carbonate.

The enzyme assay for CA was similar to that of Rickli et al. (1964). Aliquots of 10-20 µl of sample were added to 75 µl of a Tris-phenol red solution (pH 8.3), and then 50 µl of substrate (CO_2 saturated solution, pH < 4) was added, all maintained at 4°C. Upon adding the substrate, timing with a stopwatch began. The reaction was followed by the color change from red to yellow-orange (pH 6.4). Blanks with heat denatured protein and samples plus acetazolamide were used for comparison with the catalyzed reaction. Reaction volumes were adjusted to take about 15-30 seconds, whereas the blanks took about 120 seconds. Detecting the color change is subjective, but reproducible.

Activity units = (1/tc - 1/tu) x 10 tc= catalyzed reaction, tu= uncatalyzed reaction

Results

To reduce likely enzyme contamination from epithelial cells, the hard coral, *Astrangia astrangia* was cleaned, washed, and twice treated with both 0.5% SDS and 1.5% sodium hypochlorite before and after grinding the exoskeleton to a fine powder with the Thomas-Wiley Laboratory Mill using a screen size <1mm. Following EDTA decalcification of the exoskeleton of *A. astrangia,* six individual samples were found to consist of 0.07%, 0.04%, 0.10%, 0.08%, 0.17% and 0.11% matrix respectively (Table 1). The balance of the exoskeleton was primarily calcium carbonate. The total water soluble proteins (WSP) in the exoskeleton of *A. astrangia* was 10.6, 7.1, 22.4, 6.0, 22.6 and 5.3 mg/100g exoskeleton whereas percentages of the carbonic anhydrase (CA) in the WSP was 1.39%, 2.89%, 1.89%, 1.35%, 0.91% and 1.84% WSP, respectively (Table 1). The average quantity of organic matrix, WSP, and CA in 100g-coral exoskeleton is 95, 12, and 0.2 mg. Because there is so little matrix in exoskeleton, the relative amount of CA and WSP in the exoskeletal matrix is high.

Three proteins were isolated from the exoskeleton of *A. astrangia,* by affinity chromotagraphy. The proteins came off the affinity chromatography column at the same position that the bovine carbonic anhydrase elutes. All of the CA proteins were eluted off the column by KSCN (0.4M). The ligand, 4-aminomethylbenezenesulfonamide, on the column binds specifically to the carbonic anhydrase or the sulfonamide and therefore provides strong supporting evidence that the *A. astrangia* proteins are isozymes of CA.

The *A. astrangia* proteins eluted from the affinity chromatography columns were concentrated and molecular mass calculated by SDS/PAGE (10% gel). Three proteins

that were detected on the 10% gels appeared to be three isozymes for carbonic anhydrase (Table 2 and Figure 1), and had approximate molecular masses of 31, 55, 63 kDa. Molecular masses were estimated by comparing the Rf values of the proteins with those of standard MW proteins. The protein staining most intensely with comassie blue was the isozyme of 55kDa. The more intense the staining may indicate that this protein is the most abundant of the three isozymes.

Even though only small quantities of protein were isolated and the purification procedure was lengthy and somewhat harsh, enzyme activity was detected in extracts of all exoskeletons of *A. astrangia* (Figure 2). The mean coral enzyme activity was 0.227 U/mg – protein or 227 U/g – protein (see method and materials). The CA inhibitor acetazolamide (100μM) reduced CA activity of the *A. astrangia* enzyme as much as 95%. Through serial dilutions (0.001, 0.0001, 0.00001, and 0.000001 M) it was shown that acetazolamide reduced CA enzyme activity by 20%, 47%, 70%, and 95% of the uninhibited activity (Fig.2). The assay was based on pH changes by timing the visual color change of phenol red from red (pH 8.3) to orange (pH 6.4), or by taking electrostatic measurements with a pH meter. Because the assay was conducted on the concentrated extracts from the affinity column, it is not known if all three CA like proteins/isozymes were associated with the enzyme activity. The three isozymes were not individually isolated and assayed. However, the western blot data (see below) indicated a positive response to antibodies of Bovine CA II for two isozymes.

Further evidence that the affinity chromatography isolated proteins are a form of CA was the result of the enzyme-linked immunosorbent assay (ELISA) using a rabbit antibody to bovine carbonic anhydrase II (Chemicon #AB1243). The ELISA (for the

combined sample of these proteins) gave positive results to the bovine carbonic anhydrase II antibody when both CA was diluted and when the antibody was diluted (Fig 3). A Western Blot of the affinity chromatographically isolated protein fraction was also run, and showed two bands at 31 and 59kDa respectively when compared to a protein standard (Table 3 and Figure 4). The data from the Western Blot, ELISA, CA enzyme activity, CA enzyme inhibition with acetazolamide, and removal of any cells and their residue with SDS and sodium hypochlorite support the contention that CA is secreted extracellularly with the matrix proteins. For the first time the extracellular calcarious layer, purged of epithelial tissue, has been shown to have carbonic anhydrase in the matrix. It is not known why the SDS/PAGE gels indicate three proteins at 31, 55, and 63kDa while the western blot shows two proteins at 31 and 59kDa. The 59kDa protein is exactly in between the 55 and 63kDa proteins, which were detected on the gels so, it could be possible that the protein may not have been completely denatured or may be a dimer.

Table 1. Total soluble protein (EDTA extraction) and CarbonicAnhydrase (CA) in coral, *A.astrangia*

CORAL SAMPLE	TOTAL (WSP)* (mg/100g-coral)	CARBONIC ANHYDRASE ug/100g-coral (% of WSP)**	PROTEINS*** DETECTED (kDa)
Coral 1 (exoskeleton=100g) (matrix=0.07g)	10.6	146 (1.38%)	Not Determined 31, 55, 63 (SDS gel)
Coral 2 (exoskeleton=100g) (matrix=0.04g)	7.1	206 (2.90%)	31, 59 (Western Blot) 31, 55, 63 (SDS gel)
Coral 3 (exoskeleton=100g) (matrix=0.10g)	22.4	423 (1.89%)	Not Determined 31, 55, 63 (SDS gel)
Coral 4 (exoskeleton=100g) (matrix=0.08g)	6.0	81 (1.35%)	Not Determined Not Determined
Coral 5 (exoskeleton=100g) (matrix=0.17g)	22.6	206 (0.91%)	31, 59 (Western Blot) 31, 55, 63 (SDS gel)
Coral 6 (exoskeleton=100g) (matrix=0 11g)	15.3	283 (1.85%)	Not Determined 31, 55, 63 (SDS gel)
MEAN+/-STD	14 +/- 7	224+/- 108 (1.71%)	

*Total coral water soluble protein ; **Carbonic anhydrase as a total % of coral water soluble protein. Protein isolated by affinity chromatography, Mr estimated with SDS/PAGE

TABLE 2. Mr of *A. astrangia* CA and Mark 12 Standard on SDS/PAGE gel (10%). The band at 31kDa is Bovine CA and the bands at 55kDa and 63 kDa appear to be dimers. Each sample size is 10µl.

Mr of SAMPLE	Mr of SAMPLE	Mr of SAMPLE	Mr of STANDARD
63776.9	63474.1	63776.9	200000
54952.6	54381.7	55142.9	116300
31259	30703.1	31444.3	97400
			66300
			55400
			36500
			31000
			21500
			14400
			6000
			3500
			2500

Figure 1 SDS/PAGE gel with Mark 12 Standard and A. astrangia CA bands at 31, 55, and 63 kDa. The 55 kDa band stains most intensely indicating that this protein is the most abundant of the three isozymes.

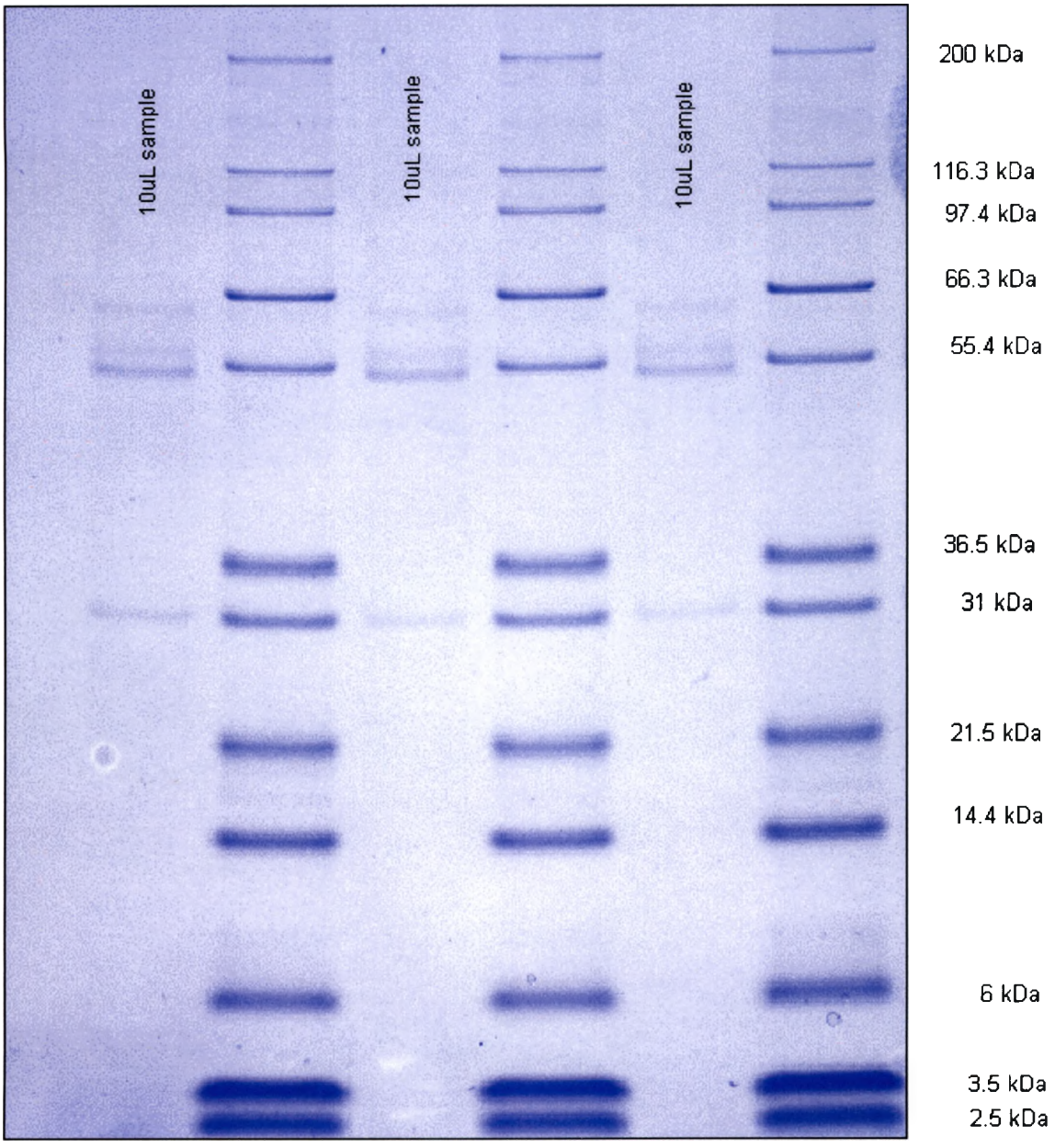

10uL sample
10uL sample
10uL sample
200 kDa
116.3 kDa
97.4 kDa
66.3 kDa
55.4 kDa
36.5 kDa
31 kDa
21.5 kDa
14.4 kDa
6 kDa
3.5 kDa
2.5 kDa

Figure 2 Inhibition of *A. astrangia* extracellular Carbonic Anhydrase by Acetazolamide

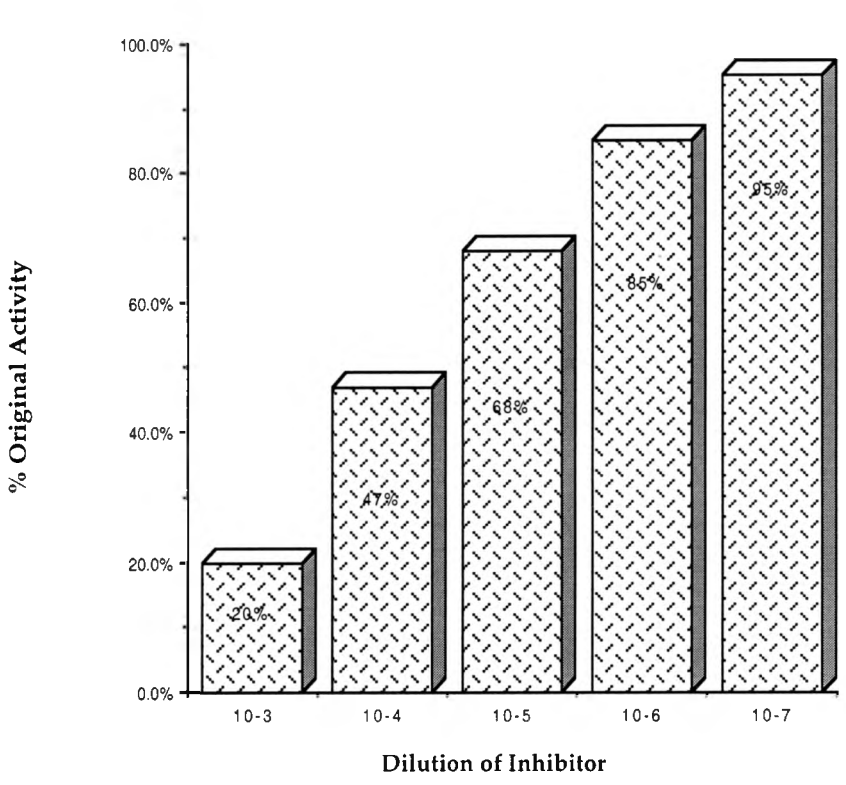

100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
% Original Activity
20%
47%
68%
85%
95%
10-3
10-4
10-5
10-6
10-7
Dilution of Inhibitor

Figure 3 The Effects of Carbonic Anhydrase Dilutions on the Cross Reaction with Antibodies to Bovine RBC Carbonic Anhydrase II.

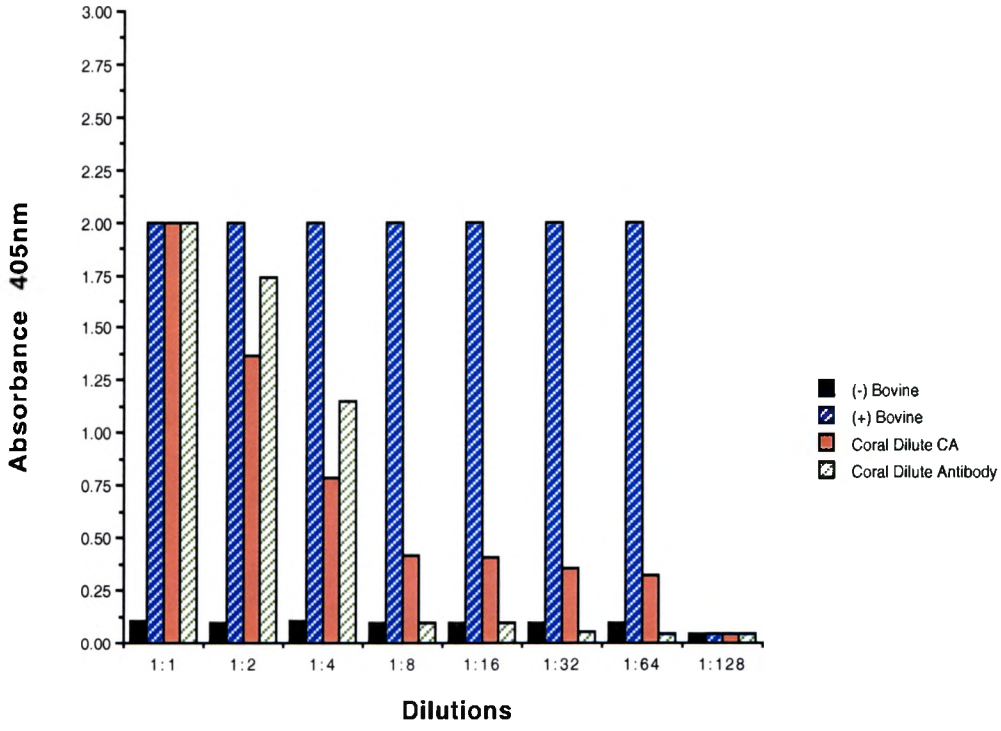
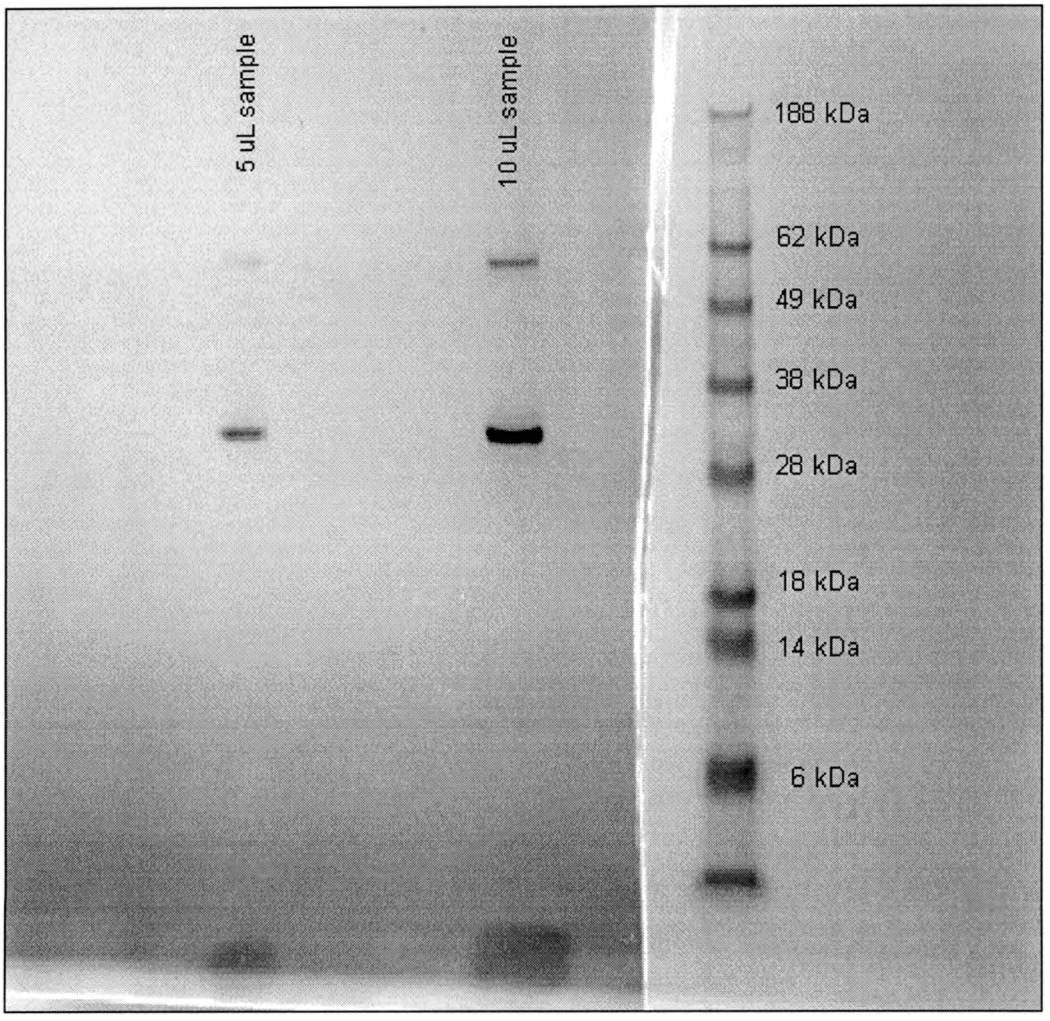

Absorbance 405nm
3.00
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
1:1
1:2
1:4
1:8
1:16
1:32
1:64
1:128
Dilutions
(-) Bovine
(+) Bovine
Coral Dilute CA
Coral Dilute Antibody

TABLE 3. Mr of *A. astrangia* CA and pre-stained standard Novex See-Blue on Western Blot analysis. The band at 31 kDa is Bovine CA and the band at 59 kDa appears to be a dimer. The first sample is 5µl and the second sample is 10µl.

Mr of SAMPLE	Mr of SAMPLE	Mr of STANDARD
59000	59500	188000
31611.1	31888.9	62000
		49000
		38000
		28000
		18000
		14000
		6000

Figure 4 Western Blot analysis with Novex See-Blue standard and *A. astrangia* CA bands at 31 and 59 kDa. The standard was cut away prior to development of samples to avoid antibody interference on the standard.


5 uL sample
10 uL sample
188 kDa
62 kDa
49 kDa
38 kDa
28 kDa
18 kDa
14 kDa
6 kDa

Discussion

The coral exoskeleton of *A.astrangia*, has three forms of CA, which were isolated and purified via affinity chromatography. Before initiation of the extraction and purification process the coral was purged of any external cell debris by washing with 0.5% SDS and 2.0% sodium hypochlorite. Any cell debris or proteins not entombed in the calcite were denatured. An oxidizing agent like hypochlorite could enhance dimerization by oxidation of sulfhydral groups if it contacted the protein, but this is unlikely for calcite entombed CA. There was no evidence for denaturation of the calcite entombed proteins by penetration of the dilute wash solutions. Dissolution of calcium carbonate with EDTA released the extracellular CA entombed in the calcite. Molecular weight estimates using SDS/PAGE gels showed one protein with a molecular weight of 31 kDa, which is similar to that of vertebrate tissue CAs (Wistrand, 1984.). Two other proteins with larger molecular weights of 55 kDa and 63 kDa were more like those detected in oyster pearls and shell with weights of 60 kDa (Miyiamoto et al., 1996; Samata et al., 1999, and Kono et al., 2000). It is likely that the two larger proteins were formed from the smaller protein via dimerization. By keeping the extraction solutions under reducing conditions with 2-mercaptoethanol, the problem of dimerization can be diminished. Krampitz et al. (1974) reported chicken CA to be sensitive to oxidizing conditions and that enzyme activity was diminished without a thiol reducing agent in the extraction solutions. Increasing or decreasing the quantity of 2-mercaptoethanol altered the ratio of the larger proteins to the smaller one. Thiol reducing agents divided a 54 kDa rabbit CA into two components, one 30 kDa and the other 24 kDa (Shafer and Dietsch, 1984). Greatest enzyme activity was detected with the 30 kDa molecule. Many elasmobranch, amphibian, reptilian and

avian, but not mammalian and teleosts CAs require reduced cysteines for activity (Sanyal, 1984). The different quantities of the three coral CA isozymes in each of the isolations (Table 1) and their activities seem to be associated with reducing conditions and dimerization.

Each sample was obtained based on exoskeletons of coral from one colony of polyps from the same gene pool. Although the total WSP from different areas of the coral and at different stages of growth could vary significantly, control of these factors was beyond the scope of this study. The entire coral consisting of both active and inactive regions was used; so entombed enzymes came from both areas. The coral had been dead for several months. CA isozymes make up 224 ± 108 μg-protein/100 g exoskeleton or 1.71%(n=6) of the total WSP of the coral exoskeleton. There is much less organic matrix in the hard coral (95 mg/100 g-exoskeleton) than there is in crustaceans (15-17 g/100 g-exoskeleton) and clams (1-2 g/100 g-exoskeleton).

The coral enzyme activity was 0.23 U/mg – protein or 227 U/g – protein and the CA inhibitor acetazolamide reduced CA activity to 95% of the uninhibited activity (Fig.2). Since the assay was conducted on concentrated extracts from the affinity column, it is not known if all of the three CA like proteins/isozymes were associated with the enzyme activity. Future studies are proposed to answer this question.

For comparison, the soft octocoral, *Leptogoria virgulata*, had only one form of CA, with a CA specific activity of 57 U/g – protein. The enzyme also was inhibited by acetazolamide (Lucas and Knapp, 1996). The data suggest that the stony coral, *A. astrangia,* has about four times the CA activity as the soft coral. So clearly CA plays an important role in the calcification of the acellular coral exoskeleton.

Antibody experiments with ELISAs and Western blots showed that the coral *A. astrangia* has CAs that are similar to bovine CA II since bovine serum CA antibodies cross reacted with the coral proteins (Lucas and Knapp, 1996).

Miyamoto et al. (1996), Samata et al. (1999) and Kono et al. (2000) have reported nacrein/CA isozymes in acellular pearls and oyster shells, but have only detected CA enzyme activity in mantle tissues and not in pearls or shells (Miyamoto et al., 1996). The data presented here, such as purification of entombed CA from coral calcite, CA enzyme activity, CA enzyme inhibition with acetazolamide, and the positive response of the coral CA to bovine CA II antibodies, add support to our contention that an extracellular coral CA plays a major role in calcification and is secreted extracellularly along with the other matrix proteins.

Protons released during the formation of carbonate ions from bicarbonate could be buffered by the available excess of bicarbonate ions maintained by CA either intracellularly or extracellularly. Carbonic acid could then be rapidly neutralized by dehydration to carbon dioxide and water via CA with carbon dioxide being ultimately removed by diffusion or photosynthesis. As concentrations of carbon dioxide, carbonic acid or bicarbonate ion change, CA could maintain the equilibrium in either direction – thus forming carbon dioxide or bicarbonate ion as directed by equilibrium conditions. If most of the carbonate ion used in coral is environmental in origin as in the soft octocoral *L. virgulata* (Lucas and Knapp, 1996) or due to zooanthellae, then an extracellular CA would be especially important in coral calcification.

Along with other matrix proteins known to initiate mineralization (Falini et al., 1996; Belcher et al., 1996; Samata et al., 1999; Kono et al., 2000), the highly ordered and

site directed biomineralization process that continuously calcifies coral also might be in part directed by extracellular CA. That matrix fibers also perform an important role in the type of crystal (argonite, calcite, or vaterite) formed in the exoskeletons has been well established in crustaceans and corals (Prosser, 1973; Williams, 1984; Falini et al., 1996; Belcher et al., 1996; Miyamoto et al., 1996). In corals, the older calcareous masses are composed of calcite, while the younger calcareous exoskeletons are primarily aragonite. Initiation of calcite formation requires supersaturation of calcium carbonate via ion pumping into the endoderm where they leave and diffuse through to the mesoglea. Calcioblasic cells of the ectoderm actively transport the ions extracellularly to the matrix. Crystal growth would then continue as long as both calcium and carbonate ions were available. As one of the components of the organic matrix, CA might assist initiation of mineralization by indirectly maintaining the availability of carbonate ions at the mineralization site. An extracellular CA could sustain high environmental bicarbonate concentrations by maintaining equilibrium conditions, which in turn would maintain the uncatalyzed bicarbonate/carbonate equilibrium, which supplies carbonate. Once the solubility product for calcite formation is exceeded, and mineralization starts in the ectoderm, where the calcite crystals would grow without additional CA, which would become entombed in the mineral. But when CA becomes buried with the matrix during crystallization and carbonate ions are depleted, secretion of more matrix proteins would be necessary for mineralization to continue, thus creating a laminated pattern. Calcification might stop if CA was not secreted along with the matrix, as occurs in the calcioblastic epidermis which is not calcified (Simkiss and Wilbur 1989).

Conclusion

In conclusion the data supports the contention that CA is excreted with the extracellular matrix in addition to working intracellularly. CA appears to have a direct effect on the calcification of corals. There were three extracellular isozyme forms of CA with molecular weights of 31, 55, and 63 kDa found in coral. Western Blot analysis showed two isozymes to be active forms reacting to antibodies of bovine carbonic anhydrase II. The coral exoskeleton also yielded high activity and CA isozymes were inhibited by acetazolamide. An ELISA was conducted on CA enzyme isolated via affinity chromatography which also gave a positive result in the cross reaction with bovine CA II antibodies. These results as well as the removal of all visible debris using sodium hypochlorite (2.0%) and SDS (0.5%), support the finding that CA is excreted extracellularly with the coral matrix.

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