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Characterization and the role of carbonic anhydrase activity in Microalgae

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Abstract: *The ability of photosynthetic organisms to utilize CO_2 for photosynthesis depends in part on the properties of RuBisCo and carbonic anhydrase. Conversion of CO_2 for the synthesis of chemicals by photosynthetic organisms is an attractive target for establishing independence from fossil reserves. Purified carboxysomes were isolated from Synechococcus sp. and Fischerella muscicola with the MW of 29 to 30 KDa. Kinetic study on carbonic anhydrase activity was performed and the total carbonic anhydrase activity was maximum in Synechococcus sp. and minimum in Fischerella muscicola. Carbonic anhydrase activity of purified protein was comparatively higher and Synechococcus sp. exhibited higher protein activity (4280 WU) in all three extracts analyzed. High proportion of amino acid lysine in carbonic anhydrase indicated the presence of an active site which enhances the carbon fixation. Therefore, understanding the CCM in terms of RuBisCo and Carbonic anhydrase activity can help in screening of efficient isolates for carbon sequestration.*

Keywords: *Carbonic anhydrase, Carboxysomes, Fischerella muscicola, RuBisCo and. Synechococcus sp.,*

I. Introduction

In cyanobacteria, for the carbon concentrating mechanism to function, there is an absolute requirement for carbonic anhydrase activity within the carboxysome to convert the bicarbonate to carbon dioxide, and a simultaneous requirement that minimal carbonic anhydrase activity be found within the cystol. The CAs are ubiquitous metallo enzymes (mainly Zn) that catalyzes the quick reversible hydration reaction of CO_2 to $HCO3^-$ and protons (H^+) or vice versa. This "reverse" reaction gives CA its name, because it removes a water molecule from carbonic acid. Due tothe vital biocatalyst role of CAs, nature has advanced its catalytic ability as one of the fastest of all enzymes to hydrate carbon dioxide and dehydrate bicarbonate a number of times (turnover number ≥104–106 reactions per second) (Donaldson and Quinn,1999). The gamma-carbonic anhydrase activity of the carboxysome structural protein CcmM from the cyanobacterium, *Nostoc* PCC 7120, was identified and characterized.This was the first time that gamma-carbonic anhydrase (CA) activity was observed in CcmM from a mesophilic cyanobacteria, a protein that was initially considered to be catalytically inactive. Using molecular and biophysical techniques, they identified the CA activity in the whole cell extracts, localized it to the carboxysome enriched fraction and provided the first kinetic characterization of the CA activity of the redox regulated amino terminal domain of protein.Algae's ability to capture CO_2 as bicarbonate in ponds thereby reducing atmospheric CO_2 emissions and reduced competition for land, particularly arable land used for food production is an added advantage. Therefore, the present research work focused on the isolation and purification of carboxysomes from the selected fresh water cyanobacteria isolated from the water bodies of Madurai.

II. Results And Discussion

2.1. Extraction of Carboxysome from *Synechococcus* sp. and *Fischerella muscicola* (So *et al*., 2004)

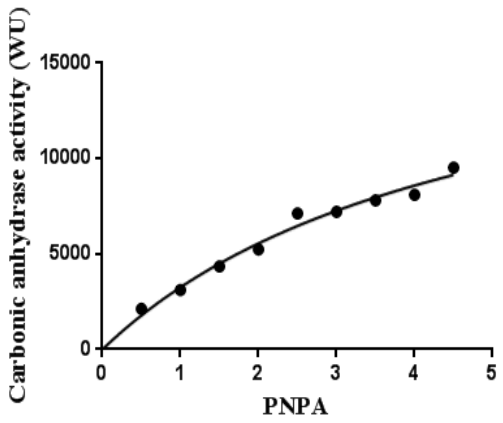
Microalgal strains of *Synechococcus* sp. and *Fischerella muscicola* at an exponential phase were resuspended in 50 ml of TEMB buffer. Purified carboxysomes were disrupted by freezing the pelleted carboxysomes at -20°C for 30 minutes. The pellet was subsequently thawed, resuspended in 100 to 200 µl of TEMB at 4°C. The resulting pellet consisted mainly of carboxysome shell "ghosts," while the supernatant contained most of the RuBisCo and carbonic anhydrase that originally had been packaged in the intact particles. Carboxysomal protein was run in SDS-PAGE for protein profile pattern. Protein bands with molecular weight of 29 – 30 kDa corresponding to carbonic anhydrase and other carboxysomal shell protein bands were visualized. Both strains of *Synechococcus* sp. and *Fischerella muscicola* showed more number of bands due to over expression of proteins. Most of the bands were noted in the higher molecular weight region.

2.2. Kinetic study on carbonic anhydrase assay activity (Armstrong *et al*., 1996)

Carbonic anhydrase activity was determined in 50 µl of extracted carboxysomal protein from *Synechococcus* sp. and *Fischerella muscicola*. Tris sulfate buffer (1.95 ml) and 1ml of PNPA reagent was added to the carboxysomal protein. An increase at A348 nm was recorded for approximately 5 minutes. The absorbance at 348nm/minute was obtained using the maximum linear rate for both the samples and the blank. One Wilbur-Anderson (W-A) unit have been defined as a drop in pH from 8.3 to 6.3 per minute at 0°C in veronal buffer. Total carbonic anhydrase activity was maximum in *Synechococcus* sp. (506 WU) and minimum in *Fischerella muscicola* (450 WU). The kinetic constants (Km and Vmax) were calculated for *p*-nitrophenyl acetate and CO_2 as substrate for carbonic anhydrase using the program Prism ver. 5.0 (Capassoa *et al*., 2012). Carbonic anhydrase activity at ten different *p*-nitrophenylacetate (p-NpA) concentration (0.125 to 5.0 mM) was determined at 0°C by measuring an increase in absorbance at 348 nm. The Michaelis-Menten constant (K_m and V_{max}) was calculated at pH 7.6. The carbonic anhydrase concentration in the assay was 300 ng. Vmax determined from the Michaelis–Menten data was plotted. Vmax was maximum in *Synechococcus* sp. for PNPA and CO2 as substrate. Km of 11mM and an enzyme purity of 96% was obtained in *Synechococcus* sp. *Synechococcus* sp. showed a Vmax value of 18907 units, and *Fischerella muscicola* (7208 units) with Km value of 4.8 mM, 0.90 mM respectively for PNPA as a substrate.

Fig: 2.3. Effect of PNPA substrate on Carbonic anhydrase assay activity

Synechococcus sp.	*Fischerella muscicola*



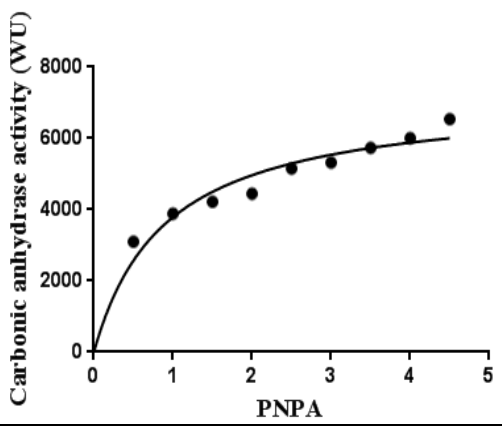
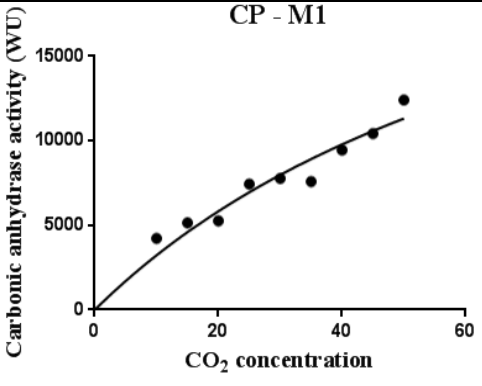
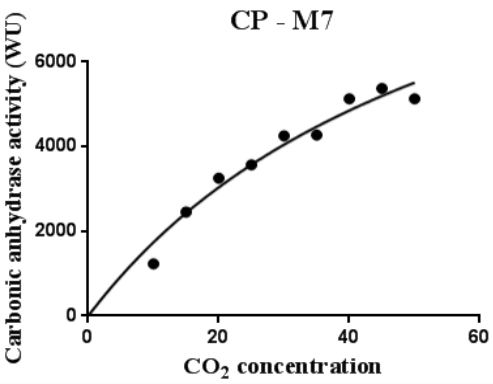


Fig: 2.4. Effect of CO_2 substrate on Carbonic anhydrase assay activity

Synechococcus sp.	*Fishcherella muscicola*





To determine Michaelis- Menten constants, increasing concentrations of the CO_2 was used as a substrate. The reaction was started by adding 30 ng of carboxysomal protein. The probe of a standardized pH meter was inserted into the test vials. The time (T) required for the pH to change from 8.3 to 6.3 was recorded. If the pH dropped below 8.3 before the pH probe was placed in the reaction mixture, the time was recorded immediately upon addition of the carbonic anhydrase. The blank measurement was repeated after running the test vials. *Synechococcus* sp. (30301 units) showed higher Vmax and Km value of 83% for CO_2 as a substrate.

$WAU = t_0 / t - 1$

where t0 and t were the measurements taken for the carbonic anhydrase without free buffer (control) and for the buffer containing the sample, respectively.

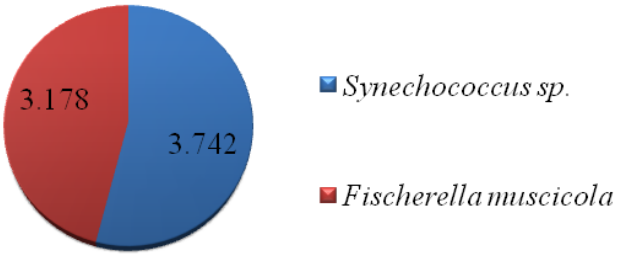
$$\text{Units/mg enzyme} = \frac{(A_{348}\text{nm/min Test} - A_{348}\text{nm/min Blank})\ (1000)}{(5.0)\ (\text{mg enzyme/ml RM})}$$

1000 = Conversion to micromoles
5 = Millimolar extinction coefficient of p-nitrophenol at pH 7.6 at 0°C
RM = Reaction Mix

2.5. Characterization of carbonic anhydrase

Concentration of zinc was measured in Atomic Absorption Spectrometer (Atkins *et al.,* 1972). Carboxysomal protein solution containing about 1 mg of protein was taken from the *Synechococcus* sp. and *Fischerella muscicola*. Carbonic anhydrase enzyme is associated with zinc where elution of metal coincides with enzyme activity. Carbonic anhydrase contains about 0.31 to 0.34% zinc moiety. The observed value was then related to the concentration of the enzyme in the stock solution. The zinc content was maximum in the CA extract of *Synechococcus* sp. corresponding to 3.742 mg/L zinc ion/subunit weight of 29,000.

Figure 2.6 Zinc concentration in carboxysomal protein (Conc./ppm)



2.7. DEAE-cellulose Ion Exchange Chromatography (Hiltonen *et al*., 1995).

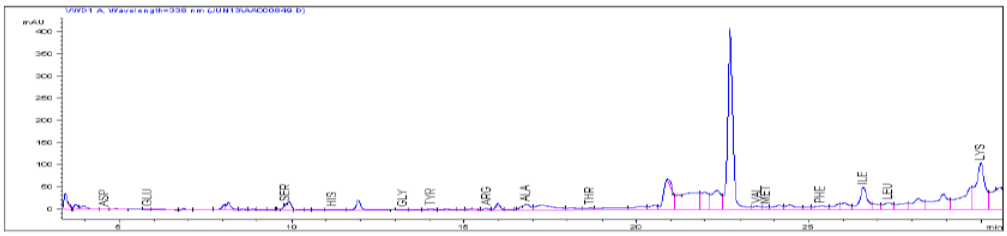
Carboxysomal protein was purified from the selected *Synechococcus* sp. and *Fischerella muscicola* using ion exchange chromatography. The active fractions were pooled, assayed for carbonic anhydrase activity, protein content and used for further characterization studies. Carbonic anhydrase activity of total protein, partially purified carboxysomal protein and purified carbonic anhydrase were compared. Carbonic anhydrase activity of purified protein was comparatively higher and *Synechococcus* sp. exhibited higher protein activity (4280 WU) in all the three extracts analyzed.

Table 2.8. Purification chart of carbonic anhydrase

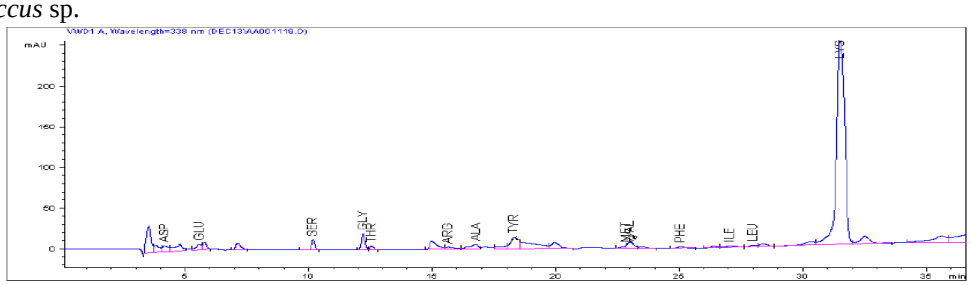
	Total protein (mg)	**Total activity (Units)**	**Specific activity (Units/mg)**	**Yield (%)**	**Purification (fold)**
Partially purified protein	***Synechococcus* sp.**				
	459	2,500	5.4		
Purified by DEAE-cellulose chromatography	532	4,280	8.04	85.6	1.4
Partially purified protein	***Fischerella muscicola***				
	372	1,150	3.09		
Purified by DEAE-cellulose chromatography	425	1,812	4.26	78.8	1.38

Amino acid profile of purified carbonic anhydrase (Huesgen, 1999) for both *Synechococcus* sp. and *Fischerella muscicola* was estimated using pre-column orthopthalaldehyde derivatization for HPLC analysis. The amino acid composition of the cyanobacterial carbonic anhydrase and carbonic anhydrase standard were subjected to regression analysis. High proportion of amino acid lysine in carbonic anhydrase indicated the presence of an active site which enhances the carbon fixation. Limitations in the concentration of sulphur containing amino acids such as methionine and absence of cysteine will indirectly affect the photosynthetic processes.

Fig : 2.9. RP-HPLC analysis of carbonic anhydrase



Synechococcus sp.



Fischerella muscicola

III. Conclusion

Carboxysomal protein was purified from the two cyanobacterial isolates namely *Synechococcus* sp. and *Fischerella muscicola* based on the photosynthetic rate, RuBisCo and carbonic anhydrase activity. The kinetic constants (K_m and V_{max}) were calculated to confirm the concentration of substrate which react with the various protein content are considered the most important factors inducing CA activity. Increasing the purity of carbonic anhydrase was achieved through DEAE cellulose chromatographic separation of carbonic anhydrase from *Synechococcus* sp. and *Fischerella muscicola*. High proportion of amino acid lysine in carbonic anhydrase indicated the presence of an active site which enhances the carbon fixation. Carbonic anhydrase enzyme is associated with zinc where elution of metal coincides with enzyme activity. According to the results obtained, studies on the CA activity of *Synechococcus* sp. showed a positive correlation between the CA activity and the photosynthetic rates of the microalgal carbon concentrating mechanism. This mechanism may allow higher carbon fixation and is not inhibited by other organisms to elevated environmental concentration of carbon dioxide.

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