Photosynthetic bicarbonate utilization in *Porphyra haitanensis* (Bangiales, Rhodophyta)

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Abstract **The activities of carbonic anhydrase (CA) and photosynthesis of *Porphyra haitanensis* were investigated in order to see its photosynthetic utilization of inorganic carbon source. Both intra- and extra-cellular CA activities existed in the thallus. CA inhibitors, acetazolamide (AZ) and ethoxyzolamide (EZ), remarkably depressed the photosynthetic oxygen evolution in seawater of pH 8.2 and 10.0, and EZ showed stronger inhibition than AZ. The observed net photosynthetic rate in seawater of pH 8.2 was much higher than that of CO_2 supply theoretically derived from spontaneous dehydration of HCO_3^-. *P. haitanensis* also showed a rather high pH compensation point (9.9). The results demonstrated that *P. haitanensis* could utilize bicarbonate as the external inorganic carbon source for photosynthesis. The bicarbonate utilization was closely associated with HCO_3^- dehydration catalyzed by extracellular CA activity. The inorganic carbon composition in seawater could well saturate the photosynthesis of *P. haitanensis*. The low K_m value and compensation points for inorganic carbon reflected the existence of CO_2-concentrating mechanism in this alga.**

Keywords: ***Porphyra haitanensis*, photosynthesis, bicarbonate, inorganic carbon utilization, carbonic anhydrase.**

Terrestrial plants usually utilize carbon dioxide (CO_2) in the air as their only source of inorganic carbon for photosynthesis. Whereas marine macroalgae living in seawater environments have access to both CO_2 dissolved in the seawater (molecular CO_2 and H_2CO_3) and the ionic forms of dissolved inorganic carbon (DIC), namely bicarbonate (HCO_3^-) and carbonate ions (CO_3^{2-}). CO_2 can easily pass through biological membranes when there is a gradient in concentration, the ionic species of inorganic carbon, bicarbonate, however, can only be transported by a specific carrier[1]. The interconversion among different forms of inorganic carbon in seawater is as follows:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow CO_3^{2-} + 2H^+.$$

The equilibrium of the reactions is correlated with pH and to lesser extent with the temperature and the chlorinity of the seawater[2].

In natural seawater (pH 8.2) balanced with atmospheric equilibrium, CO_2 concentration (about 0.014 mmol/L) is much lower than that of HCO_3^- and CO_3^{2-} (about 2 and 0.2 mmol/L, respectively)[1,2]. The dissolved CO_2 concentration decreases sharply with increased pH, which usually occurs near algal surface during photosynthesis. In addition, diffusion of CO_2 in seawater is about 10000 times slower than in air. Consequently, marine macroalgae might be exposed to CO_2 constraints, which can result in photoinhibition and even damage of photosynthetic apparatus[3]. However, many marine macroalgae have developed mechanisms for using the external pool of HCO_3^- as a source of inorganic carbon for photosynthesis[1,4,5]. A common mechanism in HCO_3^- utilization involves carbonic anhydrase (CA, EC 4.2.1.1) outside the plasma membrane, which catalyses the extracellular dehydration of HCO_3^-. This dehydration is followed by diffusion or active uptake of the derived CO_2[6—8]. Therefore, photosynthesis of marine macroalgae could be inhibited by CA inhibitors, such as acetazolamide, AZ[5]. Use of HCO_3^- following its direct uptake has been described in some green macroalgae[9—11]. Some red algae only use free CO_2 in seawater as their source for photosynthesis[8,12]. Study of the utilization mechanisms of inorganic carbon by macroalgae is essential to understand their photosynthesis and other physiological processes.

Porphyra haitanensis (Bangiales, Rhodophyta) is the principal species for seaweed cultivation in southern China. The external inorganic carbon supply for its photosynthesis is one of the most common considerations for cultivation on large scale. However, the mechanism of photosynthetic inorganic carbon utilization in *P. haitanensis* has not been documented hitherto. In this note, the relationship between CA activity and photosynthesis of *P. haitanensis* were studied to see whether its submerged photosynthesis depends on free CO_2 alone or HCO_3^- ions are involved.

1 Materials and methods

(i) Plant materials. Thalli of *Porphyra haitanensis* T. J. Chang et B. F. Zheng (about 3—4 weeks after seeding) were collected during November to December, 2000, from Nanao Island, Shantou, China, where it was cultivated by the pole-system in a bay with a shallow sandy bottom. The unwounded and healthy thalli were selected and sealed in plastic bags with some seawater, transported to the laboratory in dark cooler (1℃—4℃) within 4 h, and maintained in filtered seawater in glass aquaria at room temperature (18℃—22℃) and about 100 μmol • m^{-2} • s^{-1} (LD cycle 12∶12). The seawater was

aerated and renewed by half of the amount every day. Samples were used for experiments within 4 d of maintenance, this is a period during which the stable photosynthetic activity was recognized. After this period, the remains were discarded and fresh algal materials were recollected.

(ii) Preparation of the reaction media for photosynthetic measurement. Synthetic seawater (distilled water containing 450 mmol/L NaCl, 10 mmol/L KCl, 10 mmol/L $CaCl_2$, 30 mmol/L $MgSO_4$)[10] was used. CO_2-free seawater was prepared by reducing pH of the seawater to less than 4.0 with 0.1 mol/L HCl, and sparging for at least 1 h with high purity N_2 to remove all of the "CO_2". A known amount of biological buffers (from Sigma) was added to give a concentration of 25 mmol/L and the pH was adjusted as desired with freshly prepared 0.1 mol/L NaOH and 0.1 mol/L HCl. All manipulations were under N_2. The pH was measured with a pH meter (420A, Orion) fitted with a glass/calomel combination electrode. Different pH values were obtained with different buffers. MES was used for buffering at pH 6.0, TRIS at pH 8.2 and CAPS at pH 10.0. After preparation, the pH buffered CO_2-free synthetic seawater (reaction medium) was stored in stoppered glass containers at 4℃ before being used.

(iii) Measurement of carbonic anhydrase activity. Carbonic anhydrase (CA) activity was determined by the potentiometerical method according to Giordano et al.[13], i.e. by measuring the pH swift which represents the rate of CO_2 hydration. The time required for a drop of 0.4 pH units was measured at 5℃, using a chamber containing 6 mL buffered CO_2-free synthetic seawater of pH 8.2. Thalli of 0.1—0.15 g fresh weight were cut into small pieces (about 0.3×0.3 cm) with a shape sterile razor blade and washed for two times before being placed in the chamber. The reaction was initiated by injecting 1 mL CO_2-saturated distilled water (5℃). Total CA activity (internal plus external) was determined as the activity in crude extracts obtained by grinding 20—40 mg with sample buffer used for the determination of activity. The enzyme activity was expressed in enzyme units (EU) based on fresh weight using the formula: $EU = 10\times(T_b/T_e-1)$, where T_b and T_e are the times in seconds for the pH drop without and with the algal sample, respectively.

(iv) Measurement of photosynthetic oxygen evolution and the effects of pH and CA inhibitors. Photosynthetic rates were measured as O_2 evolution according to Gao[14] by using a Clark-type Oxygen Electrode (YSI Model 5300, USA) at 500 μmol·m^{-2}·s^{-1} (PAR, above light saturation point) and 20℃. The electrode was held in a temperature-controlled chamber. The thalli of *P. haitanensis* were cut into small pieces (about 0.3 cm×0.3 cm) and incubated in reaction medium under 100 μmol·m^{-2}·s^{-1} and room temperature for at least 1 h. This treatment was meant to minimize the effect of the cutting damage on the photosynthesis. About 100 mg sample was transferred to the O_2 electrode chamber containing 8 mL reaction media of different pH values (i.e. 6.0, 8.2 and 10.0). The reaction media were magnetically stirred. Dissolved inorganic carbon (DIC) concentration was adjusted to 2.2 mmol/L (a concentration representative of that of natural seawater) by adding given amount of $NaHCO_3$ stock solution. When a constant O_2 evolution rate was obtained (usually within 5—10 min), the CA inhibitor acetazolamide (AZ) or 6-ethoxyzolamide (EZ) (Sigma) was added into the chamber to a final concentration of 100 μmol/L. Stock solution of AZ and EZ were prepared with 40 mmol/L NaOH. It is generally believed that AZ cannot penetrate into the cell and inhibit only the extracellular CA[6,15], while EZ penetrates into the cell and inhibits both extracellular and intracellular CA[16].

(v) Inorganic carbon-dependent photosynthetic oxygen evolution. The inorganic carbon-dependent O_2 evolution was also measured in buffered synthetic seawater of pH 6.0 and 8.2 at 500 μmol·m^{-2}·s^{-1} and 20℃. Samples were left to photosynthesize in the chamber containing CO_2-free medium till no further O_2 evolved, which took about 20 min at pH 6.0 or about 40 min at pH 8.2. Aliquots of $NaHCO_3$ stock solution (11, 44, 176 mmol/L) were then injected into the chamber in order to create various inorganic carbon concentrations. O_2 evolution was recorded within 5—10 min after addition of $NaHCO_3$.

(vi) Estimation of pH and inorganic carbon compensation points. In the pH-drift experiment, 0.3 mg (fresh weight) thalli of *P. haitanensis* were immersed in 20 mL medium of filtered and sterilized natural seawater in sealed glass vials. The vials were then maintained in an incubator at 145 μmol·m^{-2}·s^{-1} (PAR) and 16℃. Some of the vials contained 100 μmol/L AZ. The pH values in the vials were monitored at regular time intervals. The pH compensation points were determined as the final pH values when it no longer increased.

The compensation points for inorganic carbon were estimated in buffered natural seawater of pH 6.0 and pH 8.2 with low inorganic carbon (<0.1 mmol/L). When O_2 evolution by the alga reached zero, the inorganic carbon concentrations in the seawater were determined as the compensation points of inorganic carbon. DIC was analyzed by using Shimadzu Total Organic Carbon Analyzer (TOC-5000A, Japan).

(vii) Calculation of the theoretical rate of uncatalyzed dehydration of HCO_3^-. The CO_2 concentration in seawater was estimated by using the apparent dissociation constants of carbonic acid in seawater adopted from Stumm et al.[17]. The theoretical production rate of CO_2 derived from uncatalyzed dehydration of HCO_3^- in seawater was calculated according to Johnson[18].

(viii) Statistics. The data were expressed at the mean values ±standard deviation ($n \geqslant 3$). Statistical significance of means was tested with *t*-test or one-way ANOVA at $P < 0.05$.

2 Results

(i) Carbonic anhydrase (CA) activity. CA activities in thalli and crude extracts of *P. haitanensis* were all detected by means of the potentiometeric method, and their values were 3.1±1.4 and 22.7±1.3 units·g^{-1} respectively, indicating the existence of both extracellular and intracellular CA in *P. haitanensis*. The extracellular CA accounted for 13.4% of the total activities of CA (external plus internal).

(ii) The effects of pH and CA inhibitors on photosynthetic oxygen evolution. The pH values affect the composition of inorganic carbon species in seawater. The ratio of CO_2/HCO_3^- decreases sharply with increased pH values, being 0.992, 0.006 and 0 at pH 6.0, 8.2 and 10.0, respectively. Fig. 1 shows the photosynthetic O_2 evolution rates of *P. haitanensis* in seawater of different pH values and the effects of CA inhibitors on the rates. The pH changes in seawater significantly affected the O_2 evolution rates. The rate at pH 8.2 was the greatest among the treatments, and the rates at pH 6.0 and pH 10.0 accounted for 40% and only 4% of them at pH 8.2, respectively. The external CA inhibitor, AZ, remarkably depressed the O_2 evolution at pH 8.2 and 10.0 by 81% and 93% respectively compared with control. However, AZ had no inhibitory effect on O_2 evolution at pH 6.0. By contrast, EZ inhibited O_2 evolution to a greater extent. It depressed the O_2 evolution by 30%, 89% and 99% at pH 6.0, 8.2 and 10.0, respectively. On the other hand, the uncatalyzed rate of CO_2-supply derived from HCO_3^- in 8 mL seawater of pH 8.2 and 2.2 mmol/L inorganic carbon was 2.3 nmol/s. However, the observed rate of photosynthetic O_2 evolution by *P. haitanensis* was 9.8±1.5 nmol/s (or 1.6±0.1 nmol/s with AZ), being 4.3 (or 0.7 with AZ) times of the theoretical CO_2-supply rate.

(iii) The response of photosynthetic O_2 evolution to inorganic carbon concentration. Fig. 2 shows that the photosynthetic O_2 evolution of *P. haitanensis* increased with increased DIC concentration, and reached maximum at DIC levels of 1.1 mmol/L (pH 8.2) or 0.22 mmol/L (pH 6.0). This indicated that the inorganic carbon composition of general seawater is rich enough to meet the requirement of inorganic carbon by photosynthesis in *P. haitanensis*. Table 1 shows that the half-saturation constants (K_m, the inorganic carbon concentration resulting in half of the maximal photosynthetic rate) and the compensation points for inorganic carbon were rather low. The values of K_m (DIC) and the compensation points for DIC at pH 6.0 were only 40% and 30% of the values at pH 8.2, respectively. However, when normalized to CO_2, the $K_m(CO_2)$ and the compensation point for CO_2 at pH 8.2 were 36 and 28 times lower than that at pH 6.0.

(iv) pH compensation point. In a closed system, marine macroalgae remove ions (such as HCO_3^-) from the surrounding fluid via active uptake or passive diffusion, and release metabolic products (such as OH^-). The

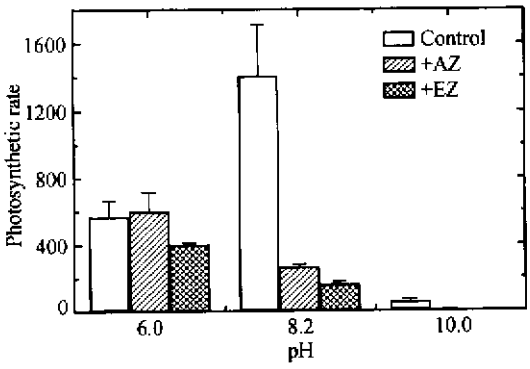

Fig. 1. The effects of pH and CA inhibitors AZ, EZ on photosynthetic oxygen evolution rates (μmol·g^{-1}·h^{-1}) of *P. haitanensis*.

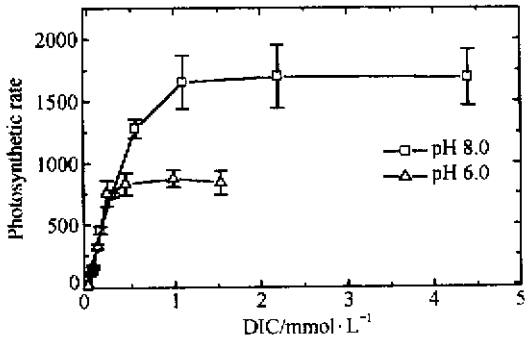

Fig. 2. The responses of photosynthetic O_2 evolution rates (μmol·g^{-1}·h^{-1}) of *P. haitanensis* to inorganic carbon concentration at pH 8.2 and 6.0.

Table 1 The apparent half-saturation values (K_m) and inorganic carbon compensation points (CCP) of *P. haitanensis* at pH 8.2 and 6.0

	K_m/μmol·L⁻¹		CCP/μmol·L⁻¹	
	DIC	CO_2	DIC	CO_2
pH 8.2	335±72	1.8±0.4	40.7±11.8	0.2±0.1
pH 6.0	137±9	67±4	12.5±3.1	6.1±1.5

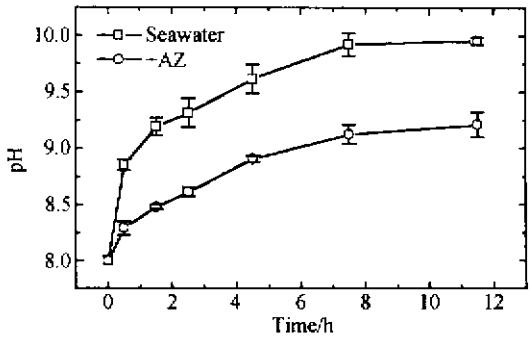

Fig. 3. The pH changes of seawater with time during the incubation of *P. haitanensis* in a closed system with or without the addition of AZ.

accumulation or depletion of differently charged ions will affect the chemistry of seawater. The pH change is the simplest and dependable indicator to measure the metabolism activity, especially for photosynthetic inorganic carbon utilization. Fig. 3 shows that the pH value of seawater in a closed system containing thalli of *P. haitanensis* increased with the incubation time till the maximal pH of 9.9 was reached. The maximal pH was 9.1 with the presence of AZ.

3 Discussion

In general, the capacities of HCO_3^- utilization in marine red macroalgae are relative lower[8,12] and their abilities to use HCO_3^- were less efficient compared to marine green and blown macroalgae[8,19,20]. Nevertheless, present study demonstrated that *P. haitanensis* was capable of using HCO_3^- as its principal external inorganic carbon source during photosynthesis.

P. haitanensis possessed CA activity, and the CA inhibitors significantly depressed its photosynthesis. Both intra- and extra-cellular CA activities could be detected by the potentiometric method, and those of the external CA accounted for more than 10% of the total CA. The proportional activity of the external CA was much greater than that reported for *P. umbilicalis*[13]. It is known that CA is involved in the utilization of HCO_3^- during photosynthesis[6—8,21,22]. The extracellular CA catalyzes the conversion of HCO_3^- to CO_2, which is taken up through the plasma membrane and then fixed in photosynthesis. In this study, the role that the CA played in *P. haitanensis* by using HCO_3^- was clearly recognized from the depression of photosynthetic O_2 evolution by the CA inhibitors. In general, seawater of pH 8.2, the photosynthetic HCO_3^- use of *P. haitanensis* catalyzed by the external CA accounted for 81% of the total inorganic carbon utilization. The intracellular CA might be located in cytoplasm and chloroplast, and their possible role lies in facilitating the transport of inorganic carbon within cells to the carboxylating site of Rubisco for final photosynthetic CO_2 fixation. As EZ could inhibit both of the extracellular and intracellular CA activities[16], EZ had much more inhibitory effect compared to AZ.

pH compensation points over 9.2 (equivalent to 0.6 μmol/L CO_2 in seawater) have been considered as an indicator of HCO_3^- utilization in macroalgae. The higher the pH compensation points, the greater the ability of HCO_3^- using[19,23]. Studies of some macroalgae have shown a clear link between pH compensation points and their $d^{13}C$ values: most macroalgae tested were able to use HCO_3^- as a source of inorganic carbon characterized with final pH values above pH 9.2 and $d^{13}C$ values between −8.81‰ and −22.55‰ A number of red macroalgae, which were dependent on CO_2 diffusion for their photosynthesis, were found to have final pH values less than pH 9.2 and more negative $d^{13}C$ values, −29.90‰ to −34.51‰[12,20]. Thus, the high pH compensation point (9.9) in *P. haitanensis* provided another evidence that this species possessed the ability of photosynthetic HCO_3^- utilization. However, at presence of AZ, the pH compensation point was remarkably decreased due to its inhibitory effect on extracellular CA, suggesting reduced extent of HCO_3^- utilization.

In general, the rates for the dissociation-association reactions, $H_2CO_3 \leftrightarrow H^+ + HCO_3^-$ and $HCO_3^- \leftrightarrow H^+ + CO_3^{2-}$, are much higher; whereas those for interconversion in hydration-dehydration reactions, $H_2O + CO_2 \leftrightarrow H_2CO_3$ and $OH^- + CO_2 \leftrightarrow HCO_3^-$, are much slower than the photosynthetic CO_2 fixation by marine algae[2]. This study indicated that the observed rates of O_2 evolution in *P. haitanensis* were much higher than the theoretical rates of CO_2-supply derived from the uncatalysed dehydration of HCO_3^-. This could be explained only by the HCO_3^- utilization. The method of comparing the theoretical rate of CO_2 supply with the observed photosynthetic rate has been used by many workers to substantiate the ability or non-ability of HCO_3^- utilization[6,12,24].

Consequently, our results showed that the HCO_3^- pool in seawater is the principal exogenous inorganic carbon source for the photosynthesis of *P. haitanensis*, and the extracellular CA-catalyzed HCO_3^- utilization was the primary mechanism to acquire inorganic carbon by this alga. This way of HCO_3^- utilization exhibits a very high capacity in general seawater of pH 8.2 due to high efficiency of external CA activity. However, it is worth noting that extracellular CA can only accelerate the interconversion between HCO_3^- and CO_2, but not affect

the CO_2 equilibrium in seawater. In addition, CO_2 generated via extracellualar CA penetrates the cells mainly by diffusion. Thus, the ability to transport of CO_2 associated with the use of HCO_3^- decreases sharply with increased pH, and it is very poor at pH above 9.5[8—10]. At the same time, very high pH reduces the inorganic carbon availability[25]. This explains the reason why the photosynthetic O_2 evolution rates of *P. haitanensis* in seawater of pH 10.0 were much smaller than those in seawater of pH 8.2. On the other hand, the rates in seawater of pH 6.0 were smaller than those of pH 8.2. This might have been caused by the lower pH in the bulk, which leads to a greater energetic cost required to maintain intracellular pH (about 7.5) or to depresse operation of ion pumps in plasmalemma.

The K_m (CO_2) for Rubisco reported for marine macroalgae was in a range of 30—70 mmol/L[26]. However, the present study showed that K_m (CO_2) for photosynthesis of *P. haitanensis* at pH 8.2 was much lower than the left margin, implying the presence of CO_2- concentrating mechanism (CCM) around the site of Rubisco carboxylation. The CA- catalyzed HCO_3^- utilization in *P. haitanensis* might be the basis of CCM[16,21,22], which plays a role in decreasing photorespiration. Additionally, *P. haitanensis* exhibited very low CO_2 compensation point (about 4.5 μL/L), much lower than that of land C_3 plants (about 40—100 μL/L), but close to that of microalgae (0.1—10 μL/L)[22], and some macroalgae such as *Ascophyllum nodosum* (5 μL/L)[27] and *Ulva lactuca* (5—10 μL/L)[28]. However, the CO_2 compensation points in some other red macroalgae, *Chondrus crispus* (35 μL/L)[29] and *Palmaria palmate* (10—35 μL/L)[12], which showed minor evidence for CCM, were higher than those of *P. haitanensis*. It is interesting to more directly measure the photosynthetic physiology of *P. haitanensis*, which could involve both HCO_3^- utilization and CCM.

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