PHOTOSYNTHETIC UTILIZATION OF INORGANIC CARBON IN THE ECONOMIC BROWN ALGA, *HIZIKIA FUSIFORME* (SARGASSACEAE) FROM THE SOUTH CHINA SEA

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The mechanism of inorganic carbon (Ci) acquisition by the economic brown macroalga, *Hizikia fusiforme* (Harv.) Okamura (Sargassaceae), was investigated to characterize its photosynthetic physiology. Both intracellular and extracellular carbonic anhydrase (CA) were detected, with the external CA activity accounting for about 5% of the total. *Hizikia fusiforme* showed higher rates of photosynthetic oxygen evolution at alkaline pH than those theoretically derived from the rates of uncatalyzed CO2 production from bicarbonate and exhibited a high pH compensation point (pH 9.66). The external CA inhibitor, acetazolamide, significantly depressed the photosynthetic oxygen evolution, whereas the anion-exchanger inhibitor 4,4'-diisothiocyanostilbene-2,2'-disulfonate had no inhibitory effect on it, implying the alga was capable of using HCO3-/CO2 as a source of Ci for its photosynthesis via the mediation of the external CA. CO2 concentrations in the culture media affected its photosynthetic properties. A high level of CO2 (10,000 ppmv) resulted in a decrease in the external CA activity; however, a low CO2 level (20 ppmv) led to no changes in the external CA activity but raised the intracellular CA activity. Parallel to the reduction in the external CA activity at the high CO2 was a reduction in the photosynthetic CO2 affinity. Decreased activity of the external CA in the high CO2 grown samples led to reduced sensitiveness of photosynthesis to the addition of acetazolamide at alkaline pH. It was clearly indicated that *H. fusiforme*, which showed CO2-limited photosynthesis with the half-saturating concentration of C3 exceeding that of seawater, did not operate active HCO3- uptake but used it via the extracellular CA for its photosynthetic carbon fixation.

Key index words: brown alga; carbonic anhydrase; CO2; inorganic carbon; *Hizikia fusiforme*; marine macroalgae; photosynthesis

Abbreviations: AZ, acetazolamide; CA, carbonic anhydrase; C3, inorganic carbon; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonate; FW, fresh weight

The dissolved gaseous CO2, [CO2]aq, is only about 12 μM, being less than 1% of HCO3- in the air-equilibrated seawater (20°C, pH 8.2, salinity 35 psu). Photosynthesis of marine algae might be CO2-limited because it diffuses slowly in water, being about 7000 times slower than in air. The photosynthetic oxygen evolution rates of many marine macroalgae, however, have been found to be faster than the theoretical maximum rates of CO2 supply from the uncatalyzed spontaneous dehydration of HCO3-/CO2 in seawater, suggesting the existence of photosynthetic utilization of HCO3- (Gao and McKinley 1994, Raven 1997). Although CO2 can easily pass through biological membranes when there is a gradient in concentration, the ionic HCO3- cannot unless actively transported by some facilitating mechanisms (Beer 1994, Axelsson et al. 1995, 1999, Larsson et al. 1997, Andrés et al. 1999). Recently, the...
external CA-catalyzed conversion of HCO$_3^-$ to CO$_2$ was suggested to be facilitated in the plasma membrane by a P-type H$^+$-ATPase (proton pump) in the brown algae *Laminaria saccharina* (Axelsson et al. 2000) and *L. digitata* (Klenell et al. 2002), the red alga *Coccolithus trunctates* (Snoeij et al. 2002), and the green alga *Cladophora glomerata* (Choo et al. 2002). The P-type H$^+$-ATPase was proposed (Schmid et al. 1996) and proved to be activated by blue light in *Laminaria* spp. (Klenell et al. 2002).

*Hizikia fusiforme* (Harv.) Okamura (Sargassaceae, Phaeophyta) is an economically important species in China, Korea, and Japan, being cultivated and used for food and alginate generation. Although its reproductive biology (Park et al. 1995, Hwang et al. 1999, Ruan and Xu 2001) and cultivation techniques (Hwang et al. 1997, Li 2001) have been studied, the photosynthetic physiology of *H. fusiforme* is poorly understood. The present work aims to clarify the mechanism of inorganic carbon (Ci) acquisition by this alga and how its plasticity may affect the use of Ci sources for photosynthesis.

**MATERIALS AND METHODS**

Material and culture conditions. *Hizikia fusiforme* (Harv.) Okamura was collected from lower intertidal rock during low tide in February and March 2002 along the coast of Nanao, Shantou, China (23°20′N, 116°35′E). The thalli were cleaned of visible epiphytes and attached sediments. Only the individuals without receptacles were selected and were transported to the laboratory in an insulated cooler (5°C) within 4 h. The samples were maintained for 3 days in a glass aquarium containing filtered natural seawater (salinity 33 psu) enriched with 45 μM NaNO$_3$ and 2.5 μM Na$_2$PO$_4$ before culture in CO$_2$ chambers (Convirion, EF7, Winnipeg, Manitoba, Canada) at 20°C and 150 μmol photons·m$^{-2}$·s$^{-1}$ (fluorescent illumination, 12:12 h light:dark cycle) with 20 (low), 360 (ambient), and 10,000 (high) ppmv CO$_2$ in aeration. The low CO$_2$ level was obtained by passing ambient air through 5 M NaOH solution, and the high CO$_2$ concentration was maintained with CO$_2$-enriched air by mixing pure CO$_2$ and ambient air. The seawater was renewed by 50% every other day. The samples were grown with the varied levels of CO$_2$ for 6–8 days before being used for experiments.

Assay of CA activity. The CA activity was assayed by the potentiometric method according to Haglund et al. (1992a,b). The time required for a drop of 0.4 pH units was measured at 2°C using a cuvette containing 6 mL buffer (40 mM Tris, pH 8.4, 5 mM EDTA-Na, 25 mM ascorbic acid, 25 mM mercaptoethanol). Thalli of about 0.25 g fresh weight (FW) were cut into segments of about 0.8-cm length with a sharp razor blade and were washed two times with the buffer before placed in the cuvette. The reaction was initiated by injecting 1 mL CO$_2$-saturated distilled water (2°C). Total CA activity was determined as the activity in the crude extract of about 50 mg fresh samples homogenized in the buffer. The enzyme activity was estimated as follows: EU = 10 × (T$\text{d}$/T$\text{d}$ - 1), where T$\text{d}$ and T$\text{d}$ are the times in seconds for the pH drop without and with the algal sample, respectively.

**pH-drift experiment.** A total of 1.0 g FW of *H. fusiforme* thalli was immersed in 20 mL filtered seawater in sealed glass vials. The vials were then maintained in an incubator at 145 μmol photons·m$^{-2}$·s$^{-1}$ and 16°C. The pH changes in the seawater were recorded with a pH meter (420A, Orion, Boston, MA, USA). The pH compensation point was determined as the point where pH no longer increased (Maberly 1990).

**Photosynthetic measurements.** CO$_2$-free seawater was prepared according to Gao et al. (1993). The biological buffers (Sigma, St. Louis, MO, USA) were used as final concentrations of 20 mM to adjust the pH in addition to using 0.5 M NaOH and HCl. Varied pH levels were obtained with MES (pH 6.5), HEPES (pH 7.5), Tris (pH 8.2 and 9.0), and CAPS (pH 10.0). Although Tris buffer has been shown to inhibit the photosynthetic carbon uptake by the marine brown macroalga *Laminaria saccharina* (Axelsson et al. 2000) and the seagrass *Zostera marina* (Hellblom et al. 2001), it did not affect the photosynthetic O$_2$ evolution of *H. fusiforme* (Table 1).

Photosynthetic rates were measured as O$_2$ evolution by using a Clark-type oxygen electrode (YSI model 5300, Yellow Spring, OH, USA) at 600 μmol photons·m$^{-2}$·s$^{-1}$ and 20°C. The irradiance was provided by a halogen lamp, and the temperature was maintained constant by using a cooling circulator (Cole Parmer, Chicago, IL, USA). Segments of *H. fusiforme* were incubated in seawater at 100 μmol photons·m$^{-2}$·s$^{-1}$ and 20°C for at least 1 h before the measurement in an attempt to minimize the effect of cutting. About 0.5 g of fresh samples were transferred to the O$_2$ electrode chamber containing 8 mL of the CO$_2$-free seawater, which was magnetically stirred. Samples were allowed to photosynthesize to deplete the remaining Ci in the cells and the bulk medium until no oxygen evolution was observed. A known quantity of 200 mM NaHCO$_3$ solution was injected into the chamber to obtain the desired levels of Ci.

AZ (Sigma) was used as an inhibitor of extracellular CA (Haglund et al. 1992a,b), and DIDS (Sigma) was used to inhibit the direct uptake of HCO$_3^-$ by the algal cells (Axelsson et al. 1995, 1999). AZ or DIDS solutions were added into the chamber to final concentrations of 200 and 400 μM, respectively. Stock solution of AZ (50 mM) was prepared in 0.05 M NaOH and that of DIDS (50 mM) by dissolving it in distilled water.

**Calculation of the maximum rate of CO$_2$ supply from uncatalyzed HCO$_3^-$ dehydration.** The theoretical maximum rates of CO$_2$ production derived from spontaneous (uncatalyzed) dehydration of HCO$_3^-$ in the seawater were calculated according to Miller and Colman (1980) and Matsuda et al. (2001), provided that the alga consumed bulk CO$_2$ at a rate causing it to approach zero. The rate of such a conversion (d[CO$_2$]/dt) could be described by the following equations:

$$\frac{d[CO_2]}{dt} = K_1 \times [DIC]/A + (K_2 + [DIC] \times [H^+] + K_{H, CO_3})/A,$$

$$A = 1 + [H^+] + K_1 + K_2/[H^+]$$

where $K_1$ and $K_2$ are the rate constants for the reactions HCO$_3^-$ → CO$_2$ + OH$^-$ and H$_2$CO$_3$ → CO$_2$ + H$_2$O, respectively. $K_{H, CO_3}$ correspondingly represent the coefficient.

**Table 1.** Net photosynthetic rates (μmol O$_2$·g$^{-1}$ FW·h$^{-1}$) of *Hizikia fusiforme* in natural seawater containing 0.55 or 2.2 mM Ci, after addition of Tris buffer (final concentration 20 mM).

<table>
<thead>
<tr>
<th>Control</th>
<th>Tris buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 8.2</td>
<td>0.55 mM Ci</td>
</tr>
<tr>
<td>7.7±0.8</td>
<td>8.0±1.5</td>
</tr>
<tr>
<td>2.2 mM Ci</td>
<td>21.8±2.8</td>
</tr>
<tr>
<td>pH 9.0</td>
<td>2.2 mM Ci</td>
</tr>
<tr>
<td>12.3±2.5</td>
<td>11.5±1.1</td>
</tr>
</tbody>
</table>

Values are means ± SD (n = 3).
RESULTS

CA activities were detected potentiometrically with both the thalli and homogenates of *H. fusiforme* grown at 20 (low), 360 (ambient), and 10,000 (high) ppmv CO₂ in aeration (Table 2), indicating the presence of both extracellular and intracellular CA regardless of the CO₂ background in culture. However, the extracellular and intracellular CA activities were reduced by about 60% in the samples grown at high CO₂ compared with low CO₂.

The rates of measured photosynthetic oxygen evolution exceeded those of theoretical maximum CO₂ supply derived from uncatalyzed dehydration of HCO₃⁻ at different levels of pH in seawater containing either 1.0 (Fig. 1) or 2.0 mM C₅, (Table 3), regardless of the CO₂ concentrations in culture. In the presence of AZ, with the extracellular CA being inhibited, the theoretical rates of CO₂ supply exceeded the photosynthetic CO₂ demands at pH 8.2 but not at pH 9.0 (Table 3). The pH in the unbuffered seawater in a sealed vial with *H. fusiforme* grown at ambient CO₂ increased with the time of incubation to reach a compensation point of 9.66 in 6 h (Fig. 2).

The rates of photosynthetic oxygen evolution by the low, ambient, and high CO₂-grown *H. fusiforme* were determined in pH-buffered seawater containing 2.0 mM C₅ with or without the addition of the inhibitors AZ and DIDS (Fig. 3). The lack of sensitivity of O₂ evolution to Tris buffer (Table 1) provided the actual photosynthetic rates of *H. fusiforme* measured here. Oxygen evolution rates were significantly (*P*<0.05) reduced by the rise of pH from 8.2 to 9.0, irrespective of the CO₂ levels in culture. Net photosynthetic rates were higher with the low CO₂ but lower with the high CO₂ grown samples, compared with those of the ambient CO₂ grown samples at pH 8.2 as well as pH 9.0. AZ had pronounced inhibitory effects on the rates at both pH 8.2 and 9.0 despite the background CO₂ levels. The inhibition was highest in the low CO₂ grown and lowest in the high CO₂ grown samples. Addition of DIDS did not affect (*P*>0.1) the photosynthetic rate irrespective of the CO₂ concentrations in culture (Fig. 3). Moreover, no effect by AZ or DIDS was found at pH 6.5 for all the samples grown at the varied CO₂ levels.

When the samples grown at the varied CO₂ levels were incubated in the seawater of varied C₅ concentrations at pH 6.5, their photosynthetic O₂ evolution appeared to be saturated at 1.1 mM C₅ (Fig. 4). The apparent half-saturation constant was not significantly (*P*>0.05) affected by the CO₂ levels in culture, but the C₅-saturated photosynthetic rate was markedly reduced in the samples grown at the high CO₂ (Table 4). When incubated at pH 8.2, a condition of lower CO₂ supply compared with that at pH 6.5, net photosynthesis appeared to be saturated at 4.4 mM C₅ in the low CO₂ and at higher C₅ concentrations in the ambient and high CO₂ grown samples (Fig. 4). Additionally, the high CO₂ grown samples showed lower O₂ evolution rates as well as lower apparent affinities for C₅ compared with those grown at ambient and low CO₂ levels (Table 4).

**DISCUSSION**

Comparison of the observed photosynthetic rates with the theoretical rates of CO₂ supply, together with the pH-drift pattern, indicated that *H. fusiforme* could use HCO₃⁻ as a source of C₅ for photosynthesis, as reported in many other marine macroalgae (Axelsson and Uusitalo 1988, Surif and Raven 1989, Maberly 1990, Haglund et al. 1992a,b, Johnston et al. 1992). It has been found that direct HCO₃⁻ uptake could be facilitated by a mechanism with similar properties to the red blood cells anion exchanger (AE1; Drechsler et al. 1993, 1994), such as being inhibited by DIDS. A DIDS-sensitive mechanism had been reported in *Ulva* spp. (Drechsler et al. 1993, 1994, Axelsson et al. 1995, Axelsson et al. 1995).
Table 3. Ratios of the measured rates of photosynthetic O₂ evolution (nmol O₂·mL⁻¹·min⁻¹) to the theoretical rates of CO₂ supply derived from uncatalyzed dehydration of HCO₃⁻ at pH 8.2 and 9.0 in the seawater containing 2.0 mM Ci for Hizikia fusiforme grown with 20, 360, and 10,000 ppmv CO₂.

<table>
<thead>
<tr>
<th>CO₂ (ppmv) in culture</th>
<th>pH 8.2</th>
<th>pH 9.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>+AZ</td>
</tr>
<tr>
<td>20</td>
<td>2.35 ± 0.11ᵇ</td>
<td>0.65 ± 0.11ᵇ</td>
</tr>
<tr>
<td>360</td>
<td>2.01 ± 0.18ᵇ</td>
<td>0.61 ± 0.04ᵇ</td>
</tr>
<tr>
<td>10,000</td>
<td>1.21 ± 0.24ᵇ</td>
<td>0.63 ± 0.12ᵇ</td>
</tr>
</tbody>
</table>

The theoretical values were calculated by assuming that the whole volume of the bathing medium was available for uncatalyzed conversion of HCO₃⁻. The concentration of AZ was 200 μM. Values are means ± SD (n = 6).

ᵃᵇᶜ Different superscripts in the same column are significantly different (P < 0.05).

Graphical abstracts and graphical data are not included in the text but are available as separate files.
photosynthesis to the addition of AZ at alkaline pH. The involvement of external CA activity in HCO$_3^-$ utilization during photosynthesis has been shown for a great number of macroalgae (Mercado et al. 1997, 1998, Raven 1997) and is also confirmed here for the commercially important *H. fusiforme*. Regulation of CA activity with a clear connection with HCO$_3^-$ utilization capacity has so far only been demonstrated in a few species, including *Fucus serratus* (Johnston and Raven 1990), *Ulva* spp. (Björk et al. 1993), and *Gracilaria tenuistipitata* (García-Sánchez et al. 1994). Mercado et al. (1997) reported no reduction in external CA activity in the red alga *Porphyra leucosticta* when cultured with elevated Ci, though its activity was increased at a lowered Ci level. No evident relationship was observed between external CA activity and the capacity of HCO$_3^-$ utilization in this alga, which was interpreted as the occurrence of a CO$_2$ transporter that worked in association with the external CA activity. In the present work, active CO$_2$-transport mechanism (CO$_2$-pump) in *H. fusiforme* was unlikely present, because the HCO$_3^-$ utilization was closely associated with the external CA activity. If photosynthesis depended on both the CO$_2$ gradient created by the external CA activity and the CO$_2$ diffusive entries, then the energy cost could be lower than if only an active CO$_2$ pump was operating (Mercado et al. 1997). In the present study, net photosynthesis of *H. fusiforme* was not enhanced at the acidic pH when it was not Ci limited, which is suggestive of the absence of active CO$_2$-transport mechanism. Therefore, it appeared to depend on CO$_2$ diffusive entry driven by the external CA-mediated CO$_2$ gradient, as suggested in *Chondrus crispus* (Smith and Bidwell 1989), *Fucus serratus* (Haglund et al. 1992b), and *Bostrychia scorpioides* (Mercado and Niell 1999).

It seems that the ability of macroalgae to use HCO$_3^-$ differ according to their zonations (Axelsson and Uusitalo 1988, Maberly 1990, Raven and Osmond 1992, Mercado et al. 1998). Surif and Raven (1989) reported that photosynthesis of eulittoral *Fucus* spp. tested was essentially saturated at the Ci level of seawater, whereas the normally submersed *Halidrys siliquosa*, *Alaria esculenta*, and *Laminaria* spp. were only about half Ci saturated in the seawater. *Hizikia fusiforme* is distributed at a lower intertidal zone; its photosynthesis was demonstrated in the present work to be Ci limited, with low Ci affinity (the half-saturating Ci level

![Image](image.png)

**Fig. 4.** Rates of photosynthetic oxygen evolution as a function of Ci concentrations in *Hizikia fusiforme* thalli grown with (□) 20, (○) 360, and (△) 10,000 ppmv CO$_2$. The seawater was buffered at pH 6.5 and 8.2. Vertical bars represent ± SD (n = 6).

**Table 4.** Ci-saturated rates of photosynthetic O$_2$ evolution (V$_{\text{max}}$) and apparent half-saturating Ci concentration (K$_{1/2}$) at pH 6.5 and 8.2 in *Hizikia fusiforme* grown at 20, 360, and 10,000 ppmv CO$_2$.

<table>
<thead>
<tr>
<th>CO$_2$ (ppmv) in culture</th>
<th>20</th>
<th>360</th>
<th>10,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 6.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{\text{max}}$ (μmol O$_2$·g$^{-1}$ FW·h$^{-1}$)</td>
<td>50.1 ± 3.7$^a$</td>
<td>44.2 ± 2.0$^a$</td>
<td>30.0 ± 2.1$^b$</td>
</tr>
<tr>
<td>K$_{1/2}$(Ci) (mM)</td>
<td>0.41 ± 0.07$^a$</td>
<td>0.55 ± 0.12$^a$</td>
<td>0.59 ± 0.15$^a$</td>
</tr>
<tr>
<td>K$_{1/2}$(CO$_2$) (μM)</td>
<td>93.9 ± 16.0$^a$</td>
<td>126.0 ± 27.5$^a$</td>
<td>135.1 ± 34.4$^a$</td>
</tr>
<tr>
<td>pH 8.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{\text{max}}$ (μmol O$_2$·g$^{-1}$ FW·h$^{-1}$)</td>
<td>58.5 ± 4.1$^a$</td>
<td>69.3 ± 16.2$^a$</td>
<td>26.4 ± 5.0$^b$</td>
</tr>
<tr>
<td>K$_{1/2}$(Ci) (mM)</td>
<td>1.62 ± 0.26$^a$</td>
<td>2.28 ± 0.26$^a$</td>
<td>3.06 ± 0.24$^a$</td>
</tr>
<tr>
<td>K$_{1/2}$(CO$_2$) (μM)</td>
<td>8.4 ± 1.4$^a$</td>
<td>11.9 ± 1.4$^a$</td>
<td>15.9 ± 1.2$^a$</td>
</tr>
</tbody>
</table>

Values are means ± SD (n = 6).

$^a,b,c$ Different superscripts in the same row are significantly different (P < 0.05).
exceeded that of seawater), albeit it was capable of using \( \text{HCO}_3^- \) in seawater.

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