Changes in the Gill Carbonic Anhydrase Activity of Fish Exposed to *Chattonella marina* Red Tide, with Special Reference to the Mortality

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The activities of carbonic anhydrase (EC 4.2.1.1) in the gills of yellowtail *Seriola quinqueradiata* and red sea bream *Pagrus major* exposed to *Chattonella marina* red tide were measured.

When yellowtail and red sea bream were exposed to the seawater containing *C. marina* of 2,900 and 6,500 cells/ml, respectively, the gill enzyme activities were rapidly decreased. After about 40 min, these were not detectable.

To simulate this phenomenon by inhibiting gill carbonic anhydrase *in vivo*, yellowtail were exposed to the seawater containing 0.2 mM acetazolamide, a potent and specific inhibitor of the enzyme, and 5 ppm Triton X-100 which was used to remove the mucus from the gills. The fish swam violently in proportion to the duration of exposure and were fatal in about 60 min. After 60 min, the oxygen partial pressure in the arterial blood was reduced to 25-40% of the pre-exposure value.

Judging from these results, the reduction of carbonic anhydrase activity in the gills may be one of the principal causes of mortality by the exposure of fish to *C. marina*.

Fishes, especially yellowtail *Seriola quinqueradiata*, exposed to the red tide seawater are known to die of asphyxia.¹⁻⁴⁾ According to Yamaguchi *et al.*,⁴⁾ the asphyxia is caused by the reduction of pH value in the blood. Results of our previous histochemical examination⁵⁾ showed that carbonic anhydrase activity decreased prominently in the gills of yellowtail exposed to *Chattonella marina* red tide. The reduction of carbonic anhydrase activity seems to decrease the blood pH, because the inhibition of its activity in the gills of some fishes is known to decrease the excretion of hydrogen ion from the gills to the surrounding medium.^{6,7)} However, our previous study did not show quantitatively and directly the reduction of this enzyme activity and thereby the decrease of oxygen uptake in the gills.

Present study was performed to determine the quantitative changes of carbonic anhydrase activity in the gills of yellowtail and red sea bream *Pagrus major* exposed to the seawater containing *C. marina*, and the decrease of oxygen uptake in the gills of yellowtail by inhibiting its activity.

Materials and Methods

Fish and Reagents

Yellowtail were obtained from fish farms in Kagoshima Prefecture (10 specimens, 830-1160 g), in Yamaguchi Prefecture (8 specimens, 532-590 g), and from Miyazaki Prefectural Fisheries Experimental Station (3 specimens, 450-590 g). Red sea bream (11 specimens, 530-690 g) were obtained from fish farms in Kagoshima Prefecture. Acetazolamide was purchased from Sigma, and Triton X-100 and *p*-nitrophenyl acetate were purchased from Nakarai Chemicals. Other reagents were of analytical grade.

Culture of *C. marina*

C. marina was collected in Kagoshima Bay in 1978, and cultured in the medium of SW-2⁸⁾ under controlled conditions.

Exposure of Fish to Seawater Containing *C. marina* and Low Dissolved Oxygen

Ten yellowtail specimens and eleven red sea bream specimens were used in this experiment.

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Two individuals of yellowtail were exposed to *C. marina* of 2,900 cells/ml for two different periods of exposure: 40 and 80 min. Two individuals of red sea bream were exposed to *C. marina* of 6,350 cells/ml for four different periods of exposure: 14, 30, 45, and 60 min. The remainder was kept in natural seawater as controls. During the experiment, the temperature and dissolved oxygen (DO) in the containers were 25°C and 5 ppm, respectively. Three yellowtail specimens were kept in another container and N₂ gas was gradually blown into the seawater to decrease DO, until these fishes were asphyxiated.

The fishes were taken up after the exposure to *C. marina* or asphyxiation and the gill carbonic anhydrase activities were measured.

Carbonic Anhydrase (EC 4.2.1.1) Assay

Gills of fish were perfused with physiological saline via heart for about 30 min and stored at -20°C for 24 h. The gills were homogenized with 0.25 M sucrose and the homogenate obtained was centrifuged at 10,000×g for 20 min. The supernatant was used as a crude enzyme solution. The enzyme activity was measured by the method of Mashiter and Morgan.⁹⁾ The 3 ml reaction mixture containing 0.8 mM *p*-nitrophenyl acetate, 0.067 M Tris-H₂SO₄ buffer (pH 7.5) and 0.1 ml crude enzyme solution, was incubated at 35°C (yellowtail) or 30°C (red sea bream). The hydrolysis reaction was followed spectrophotometrically, as the increase in absorbance due to the formation of *p*-nitrophenol. Absorbance measurement was made using a Shimadzu UV-190 spectrophotometer at 348 nm (isosbestic point for *p*-nitrophenol and *p*-nitrophenolate).¹⁰⁾ Absorbance reading was recorded automatically over a period of 10 min, so as to permit the calculation of a rate of increase in absorbance. For each assay, replicate reaction mixture was also prepared which differed from the first only in respect to a 10 min preincubation of the crude enzyme solution with acetazolamide at a concentration of 1.6 mM; the rate of increase in absorbance for this inhibited mixture was measured and subtracted from the uninhibited rate. The subsequent references to "carbonic anhydrase activity" strictly refer to acetazolamide sensitive esterase activity. However, carbonic anhydrase is readily distinguishable from other esterases, since its esterase activity is powerfully inhibited by acetazolamide, which has little or no effect on other esterases.

Measurement of Partial Oxygen Pressure in Arterial Blood of Fish Exposed to Acetazolamide and/or Triton X-100

Eight yellowtail were cannulated to the dorsal aorta with polyethylene tubes as described by Soivio et al.¹¹⁾ Two operated fish each were kept in the following seawater solutions for 60 min; 0.2 mM acetazolamide (solution A), 5 ppm Triton X-100 (solution B), 0.2 mM acetazolamide and 5 ppm Triton X-100 (solution C), and 0.1 mM acetazolamide and 10 ppm Triton X-100 (solution D). Their dorsal aortic blood samples were obtained at 0, 15, 30, 45, and 60 min through the cannulated tubes, and then the oxygen partial pressure in the arterial blood (Pa, O₂) was measured by a digital pH/blood gas analyzer model 213 (IL INC.). Because the carbon dioxide partial pressure in the arterial blood (Pa, CO₂) of fish is much lower than that of mammals,¹²⁾ Pa, CO₂ of fish exposed to these solution cannot be measured accurately by this analyzer.

Results

Changes in the Gill Carbonic Anhydrase Activity of Fish Exposed to Seawater Containing *C. marina* or Low DO

In the preliminary examination, the gill carbonic anhydrase of yellowtail and red sea bream showed the highest activity at 35°C and 30°C, respectively, and their optimum pH was 7.5.*

Table 1 shows the activity of the gill carbonic anhydrase of yellowtail and red sea bream exposed

Table 1. Changes in carbonic anhydrase activity in the gills of yellowtail and red sea bream exposed to red tide plankton *Chattonella marina*

Fish	<i>C. marina</i> concentration (cells/ml)	Time (min)	Carbonic anhydrase activity (U/g tissue)
Yellowtail	Control		1.07±0.38* ²
	Low dissolved oxygen* ¹	60	0.78±0.04* ²
	2,900	40	n.d.* ³
	"	80	n.d.* ³
Red sea bream	Control		3.00±1.16* ²
	6,350	14	1.20
	"	30	0.98
	"	60	n.d.* ³
	"	120	n.d.* ³

*¹ 2 ppm.

*² mean±standard deviation (n=3).

*³ not detectable.

* A. Kuroki, T. Sakai, and M. Endo: Bull. Fac. Agric., Miyazaki Univ., in preparation.

Table 2. Change of oxygen partial pressure in the dorsal aorta of yellowtail exposed to acetazolamide and/or Triton X-100

Solution	Body weight of fish (g)	Oxygen partial pressure (mm Hg)				
		0 min	15 min	30 min	45 min	60 min
A Acetazolamide, 0.2 mM	935	87.0	124.8	124.8	123.4	123.2
	820	86.7	104.8	108.8	98.3	104.3
B Triton X-100, 5 ppm	710	87.2	117.6	117.1	115.8	105.3
	770	86.2	100.9	89.2	97.3	107.0
C Acetazolamide, 0.2 mM Triton X-100, 5 ppm	620	73.9	89.2	46.5	19.6	18.7
	600	78.5	85.2	72.6	40.8	32.2
D Acetazolamide, 0.1 mM Triton X-100, 10 ppm	525	82.3	42.3	65.7	45.7	25.0
	640	74.3	69.7	53.3	63.7	62.6

Table 3. Effect of Acetazolamide and Triton X-100 on carbonic anhydrase in the gills of yellowtail *in vitro*

	Concentration	Relative activity (%) [*]
Acetazolamide	0.1 mM	10
	0.2 mM	0
Triton X-100	5 ppm	100
	10 ppm	100

* The enzyme was preincubated in the presence of acetazolamide or Triton X-100 for 10 min. Results are given as percentage of the control values.

to the seawater containing *C. marina* or low DO. The activity of normal red sea bream was higher than that of yellowtail. When these fishes were exposed to the seawater containing *C. marina*, the activities were rapidly decreased. After about 40 min, these were not detectable. In contrast, the activity scarcely changed in the gills of yellowtail exposed to low DO water.

Changes in Pa, O₂ of Yellowtail Exposed to Acetazolamide and/or Triton X-100

As shown in Table 2, the fish swam normally in solution A or B, whereas in solution C, the fish swam more violently in proportion to the duration of exposure, gasped frequently, and fatal in about 60 min. When the fish were exposed to solution C, Pa, O₂ was reduced to 25–40% of the pre-exposure value after 60 min. Pa, O₂ of fish exposed to solution D also decreased to 30–80% of the pre-exposure value after 60 min. In contrast, the exposure of fish to solution A or B increased the Pa, O₂ slightly.

Effects of Acetazolamide and Triton X-100 on Gill Carbonic Anhydrase Activity in vitro

Table 3 shows the effects of acetazolamide and

Triton X-100 on the activity of gill carbonic anhydrase *in vitro*. Although 5 and 10 ppm Triton X-100 did not inhibit the activity, 0.1 and 0.2 mM acetazolamide decreased the activity to 10 and 0% of the control value, respectively.

Discussion

The exposure of fish to sea water containing *C. marina* decreased prominently carbonic anhydrase activity in the gills of yellowtail and red sea bream, although the activity in the gills scarcely changed when yellowtail were exposed to low DO water.

When yellowtail was exposed to the seawater containing both acetazolamide and Triton X-100, its Pa, O₂ decreased prominently. On the other hand, when the fish was exposed to either acetazolamide or Triton X-100, the oxygen uptake in the gills did not decrease. We observed that in the fishes exposed to the seawater containing Triton X-100, mucus was slightly removed from the gills and body surfaces. Most of mucus is expected to be lost from the secondary lamellae of gills, where a large amount of carbonic anhydrase is distributed. In addition, we have already reported³⁾ that the activity of carbonic anhydrase disappeared after the removal of mucus of the gill surface in the fishes exposed to seawater containing *C. marina*. Thus the mucus on the gill surface may defend the excretory and respiratory function of gills and may prevent acetazolamide which is a potent and specific inhibitor of carbonic anhydrase from inhibiting the gill enzyme *in vivo*. Therefore, *in vivo*, acetazolamide inhibits the gill enzyme, after the removal of mucus from the gill surface, and this inhibition probably induces the asphyxia.

We have previously suggested³⁾ that the asphyxia

in the fish exposed to *C. marina* red tide is due to the decrease of gill carbonic anhydrase, that its reduction occurs after the removal of mucus from the fish gills, and that this decrease of the activity may be one of the principal causes of mortality. The results in the present study strongly support the suggestion in our previous studies.

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