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Fisheries and Aquatic Sciences

Note
Fish Aquat Sci 17(2), 269-273, 2014

Effects of Calcification Inhibitors on the Viability of the Coralline Algae *Lithophyllum yessoense* and *Corallina pilulifera*

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Abstract

Coralline algae, the algal whitening phenomenon-causing seaweeds, are characterized by calcareous deposits in the cell wall. The viability of the coralline algae *Lithophyllum yessoense* and *Corallina pilulifera* was quantitated using a triphenyltetrazolium chloride assay and eight calcification inhibitors. Among these inhibitors, ferric citrate showed the strongest inhibition of coralline algae viability. The concentrations of ferric citrate conferring 50% inhibition were 1.7 and 3.8 mM for *L. yessoense* and *C. pilulifera*, respectively. Thus, at a specific concentration and in a localized area, ferric citrate may be used to prevent the blooming of coralline algae.

Key words: Algal whitening, Anti-fouling, Calcification inhibitor, Ferric citrate, *Lithophyllum yessoense, Corallina pilulifera*

Introduction

Many rocky seashore areas of Korea and Japan are dominated by coralline algae such as *Lithophyllum yessoense* (Suzuki et al., 1998; Kim, 2000). However, as calcareous algae cover the surfaces of rocks in a pink-colored crust, the area covered by seaweed flora decreases. This algal whitening phenomenon is observed in barren ground, coralline flats, and deforested areas, and is associated with specific species of coralline algae (Tokuda et al., 1994). Since 1990, the area affected by algal whitening, which runs from south Cheju Island to the middle East Sea, has expanded (Chung et al., 1998). In this area, most of the fleshy seaweed has disappeared from the rocks because of algal whitening, which reduces food sources and spawning locations for fish and shellfish. This phenomenon is now considered a natural hazard adversely affecting marine ecosystems and damaging commercial fishing areas. Although biological (Agateuma et al., 1997; Daume et al., 1999) and physical (Masaki et al., 1984; Johnson and Mann, 1986) factors may be sufficient to prevent the recruitment of fleshy seaweeds, allelopathic bromoform (Ohsawa et al., 2001) and fatty acid (Kim et al., 2004; Luyen et al., 2009) substances inhibit the settlement or germination of seaweed spores. One approach to restore fleshy seaweed colonization in these areas is the removal or inhibition of living coralline algae. Before applying chemicals in the field, it is necessary to test the *in vitro* inhibitory activity of several calcification inhibitors against coralline algae to identify compounds suitable for coralline species inhibition in areas affected by algal whitening. Alternatively, coralline algae may be used to pre-

Received 11 December 2013; **Revised** 09 February 2014
Accepted 10 February 2014

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vent fouling by fleshy seaweeds. When a biomimetic coralline algal material is prepared, it can be used as an environmentally friendly anti-fouling material. To generate biomimetic coralline material, it is necessary to add a calcification inhibitor to the material to prevent additional attachment and the blooming of living coralline algae on the product. Thus, a calcification inhibitor can be used to remediate algal whitening and prepare a biomimetic coralline material. In this report, inhibitors were quantitatively screened using a triphenyltetrazolium chloride (TTC) assay.

Materials and Methods

Tissue preparation

The coralline algae *L. yessoense* and *Corallina pilulifera* were collected from the rocky intertidal area at Cheongsapo (35˚09′28″ N, 129˚11′47″ E), on the east coast of Busan, Korea. Stones covered with coralline algae were transported in a container of seawater to the laboratory. After rinsing well with autoclaved seawater to remove epiphytes and debris, the encrusted or non-articulated tissues of *L. yessoense* were sonicated three times with 30-s pulses of an ultrasonic water bath (low-intensity frequency of 40 kHz) to remove other microepiphytes. The tissue was then scraped off the stones using a saw and thoroughly washed at least six times by centrifugation at 1,000 *g* for 30 s (Kang et al., 2005). Articulated coralline tissues were cleaned by brushing thoroughly and sonicating (40 kHz) twice for 1 min in autoclaved seawater, and then immersed in 1% Betadine for 2 min to eliminate epiphytes (Jin et al., 1997). The articulated coralline tissues were then rehabilitated at 18°C in Provasoli's enriched seawater (PES) (Provasoli, 1968) for 1 day before use.

Tissue culture

In the tissue cultures of coralline algae, six hydroxyapatite inhibitors (alendronate sodium trihydrate, $AlCl_3$, dichloromethylene diphosphonic acid, etidronic acid, ferric citrate, and $FeCl_3$) and one bicarbonate channel blocker (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid) as calcium inhibitors were dissolved in distilled water and added to the tissue culture solution at concentrations of 10 or 1 mM, respectively. In addition, an impermeable carbonic anhydrase inhibitor (acetazolamide) was dissolved in dimethyl sulfoxide (DMSO) and added to the solution. To measure tissue viability, 25 μL of each inhibitor was added to 5 mL of PES medium containing 0.1 g of *L. yessoense* or 0.05 g of *C. pilulifera*. The mixture was cultured for 5 days at 16°C with rotation at 20 rpm under a photon flux density (fluorescent light) of 40 μmol m^{-2} s^{-1} and on a light cycle of 16-h light/8-h dark. A reference culture was prepared by mixing 25 μL of distilled water or DMSO in the same medium. After harvesting the tissues by centrifugation at 3,000 *g* for 30 s, cell viability was measured using the TTC assay. The relative viability (%) was calculated as: (S/C) × 100, where S equals the absorbance of the tissue with inhibitors and C equals the absorbance of the reference culture.

Viability assay

The TTC assay described by Park et al. (2006) was used. A total of 1 mL of 0.8% TTC solution in seawater containing 50 mM Tris-HCl buffer (pH 8.0) was added to the tissue in a microtube and incubated in the dark for 1.5 h at 20°C under a drop of mineral oil (M-3516; Sigma, St. Louis, MO, USA). The tissue was then rinsed four times by centrifugation at 3,000 *g* for 30 s with sterilized seawater. Triphenylformazan (TPF) that formed in the tissues was extracted with 0.6 mL of 0.2 N NaOH in 75% ethanol by heating for 15 min at 60°C. Next, TPF was partitioned by adding 0.6 mL of hexane followed by vortexing. After centrifugation for 1 min, the amount of TPF from the top phase was quantified by measuring the absorbance at 475 nm.

Statistical analysis

For each assay with calcification inhibitors and control samples, the experiments were repeated at least three times. The mean values of the index were compared to the control using Student's *t*-test.

Results

To determine the effects of the calcification inhibitors on coralline viability, we compared eight commercially available inhibitors, including six hydroxyapatite inhibitors, one bicarbonate channel blocker, and one carbonic anhydrase inhibitor. Each compound at 1 and 10 mM, respectively, was added to the coralline culture, and the viability of the culture after 5 days was measured using the TTC assay. For crustose *L. yessoense* tissues, reference cultures without calcification inhibitors reached an absorbance of 1.24. Ferric citrate showed an absorbance of 0.02, which corresponded to 2% relative viability at 10 mM (Table 1). At 1 mM, ferric citrate inhibited viability to 76% compared to the control. Next, $FeCl_2$ inhibited viability to 54% at 10 mM. The effects of the calcification inhibitors on the viability of articulated coralline *C. pilulifera* were also determined using the TTC assay after 5 days of culture. A reference culture lacking calcification inhibitors reached an absorbance of 1.11. Among the inhibitors, ferric citrate, $AlCl_3$, and $FeCl_3$ inhibited viability to 15%, 20%, and 29% compared to the control, respectively, at 10 mM (Table 2). Overall, ferric citrate most significantly suppressed the viability of *L. yessoense* and *C. pilulifera*. To explore treatment concentrations, we used a dose-response curve to de-

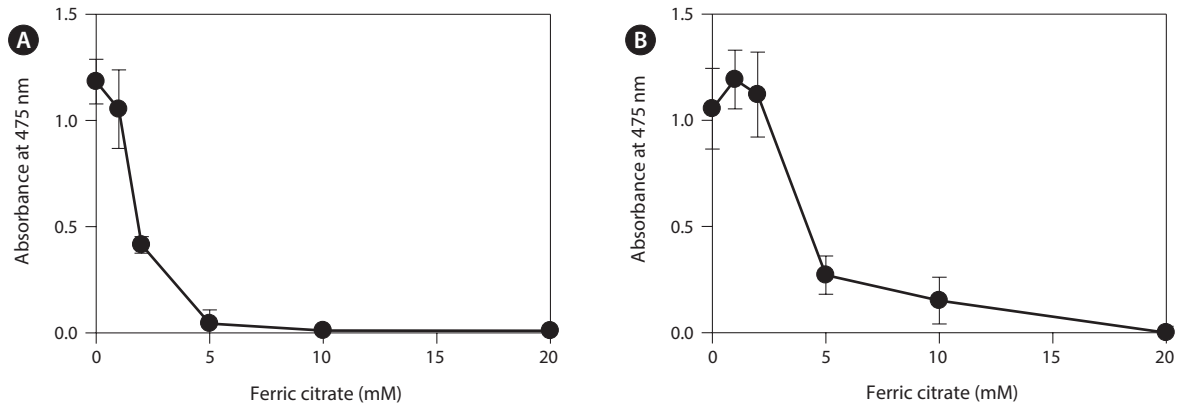

Fig. 1. Effects of ferric citrate on the viability of tissues from the crustose alga *Lithophyllum yessoense* (A) and articulated alga *Corallina pilulifera* (B). Viability was measured based on the absorbance at 475 nm; the values are expressed as the mean ± SD of at least three independent assays.

Table 1. Effects of calcification inhibitors on the viability of the crustose coralline alga *Lithophyllum yessoense*

Calcification inhibitor	10 mM		1 mM	
	Absorbance at 475 nm[a]	Relative viability[b] (%)	Absorbance at 475 nm[a]	Relative viability[b] (%)
Hydroxyapatite inhibitors				
Alendronate	0.86 ± 0.18	69	1.58 ± 0.10	127
$AlCl_3$	0.83 ± 0.18	67	1.33 ± 0.15	107
Dichloromethylene diphosphonic acid	1.27 ± 0.35	102	1.50 ± 0.06	121
Etidronic acid	0.91 ± 0.42	73	1.22 ± 0.29	98
Ferric citrate	0.02 ± 0.01	2*	0.94 ± 0.22	76
$FeCl_3$	0.67 ± 0.15	54*	1.14 ± 0.38	92
Bicarbonate channel blocker				
4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid	ND[c]	ND[c]	1.71 ± 0.31	138
Carbonic anhydrase inhibitor				
Acetazolamide	0.89 ± 0.40	72	1.18 ± 0.19	95

[a]The reference culture showed an absorbance of 1.24 ± 0.14. The data represent the mean ± SD ($n \geq 3$).
[b]The relative viability (%) is expressed as (S/C) × 100, where S is absorbance in the presence of the inhibitor and C is absorbance of the reference.
[c]ND, not determined due to solubility issues.
*$P < 0.01$.

Table 2. Effects of calcification inhibitors on the viability of the coralline alga *Corallina pilulifera*

Calcification inhibitor	10 mM		1 mM	
	Absorbance at 475 nm[a]	Relative viability[b] (%)	Absorbance at 475 nm[a]	Relative viability[b] (%)
Hydroxyapatite inhibitors				
Alendronate	0.92 ± 0.08	83	1.24 ± 0.02	112
$AlCl_3$	0.22 ± 0.10*	20	1.03 ± 0.29	93
Dichloromethylene Diphosphonic acid	0.71 ± 0.11	64	0.88 ± 0.21	79
Etidronic acid	0.43 ± 0.21*	39	0.80 ± 0.15	72
Ferric citrate	0.17 ± 0.11*	15	0.81 ± 0.19	73
$FeCl_3$	0.32 ± 0.14*	29	1.18 ± 0.19	96
Bicarbonate channel blocker				
4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid	ND[c]	ND[c]	0.61 ± 0.19	55
Carbonic anhydrase inhibitor				
Acetazolamide	0.51 ± 0.12*	46	0.93 ± 0.23	76

[a]The reference culture showed an absorbance of 1.11 ± 0.15. The data represent the mean ± SD ($n \geq 3$).
[b]The relative viability (%) is expressed as (S/C) × 100, where S is absorbance in the presence of the inhibitor and C is absorbance of the reference.
[c]ND, not determined due to solubility issues.
*$P < 0.01$.

termine the concentration resulting in 50% inhibition (IC_{50}) and the minimum concentration resulting in 100% inhibition (MIC). For ferric citrate against *L. yessoense*, a typical gradient of viability inhibition ranged sigmoidally with IC_{50} and MIC values of 1.7 and 10 mM, respectively (Fig. 1A). For *C. pilulifera*, a typical gradient of inhibition showed IC_{50} and MIC values of 3.8 and 20 mM, respectively (Fig. 1B).

Discussion

Algal whitening can devastate marine forests. However, algal whitening-causing coralline species may be used to prevent fouling by fleshy seaweed. Thus, biomimetic coralline algae may be applicable as an environmentally friendly material for anti-fouling coating treatment. To generate biomimetic coralline algae, it is necessary to include calcification inhibitors in the material to prevent the additional attachment and growth of living coralline species. Calcification is a critical process in plants because calcareous skeletons support and protect the soft parts of organisms, and secreted proteins play major roles in the photosynthetic assimilation of bicarbonate and nutrient acquisition (McConnaughey and Whelan, 1997). To identify potent inhibitors of coralline algal growth, eight commercial calcification inhibitors were compared using TTC assays. Among them, ferric citrate or Fe(III) citrate showed the strongest inhibition against coralline cell viability. Ferric citrate as a hydroxyapatite inhibitor is also known to be a non-protein-bound iron transporter in plants (Solti et al., 2012) and animals (Baker et al., 1998). Part of the ferric ion undergoes reduction to ferrous iron mediated by ferric chelate reductases (Jeong et al., 2008). Ferrous citrate or Fe(II) citrate induces oxidative damage in mitochondria through lipid peroxidation and alterations in membrane proteins (Castilho et al., 1994). Under natural conditions, iron can be present as divalent or trivalent cations depending on the chemical environment, which makes it a good cofactor for oxidoreductase-type enzymes. Nevertheless, free ferrous ions are dangerous to living organisms as they can catalyze the Fenton reaction and produce reactive radicals (Winterbourn, 1995). Thus, at specific concentrations and in a localized area, ferric citrate may be used to prevent the additional settlement of coralline algae on biomimetic materials or to prevent the blooming of coralline algae.

Acknowledgments

This work was supported by a National Research Foundation of Korea grant funded by the Korean Government (MEST) (NRF-M1A5A1-2011-0029963).

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