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A Simple Screening Method for Anti-attachment Compounds Using Monospores of *Porphyra yezoensis* Ueda

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We measured the anti-attachment activity of allelochemical and antifouling substances using monospores from *Porphyra yezoensis* Ueda as an assay. Methanol or aqueous extracts (20 μg/mL) from 32 seaweeds were added to monospore suspensions. Methanol extracts of *Corallina pilulifera, Ishige sinicola, Sargassum horneri,* and *Sargassum sagamianum* inhibited attachment by >90% compared to the reference. Phenolic compounds fractionated from *S. sagamianum* caused the most potent inhibition. *P. yezoensis* monospores also showed significant sensitivity to known allelochemical and algicidal compounds.

Key words: Allelochemical, Antifouling, Anti-attachment, Monospore, *Porphyra yezoensis*

Introduction

Porphyra yezoensis, a marine red alga, is one of the most important commercially cultivated edible seaweeds and is a valuable experimental model for fundamental and applied research in marine plants (Saga and Kitade, 2002). Monospores (blade archeospores) from juvenile blades are easily produced by the addition of allantoin (Mizuta et al., 2003) or by adjusting culture conditions (Hwang et al., 1997). Most monospores are released from blades only a few millimeters long. They germinate to produce new juvenile blades, which can then produce additional monospores. The monospores are easily maintained under laboratory conditions and reproduces throughout the year. We have developed a procedure for axenic isolation and culture of the monospores (Choi et al., 2002), which allows the monospores to be conveniently used as a target organism for an anti-attachment bioassay. Here, we report the development of a preliminary screening system for detecting allelopathic or antifouling compounds in the marine environment by using *P. yezoensis* monospores.

Materials and Methods

Monospore culture

Juvenile blades of *Porphyra yezoensis* Ueda were collected from a rocky shore at Pohang, Korea. The fresh blades were rinsed, sonicated (60 kHz) twice for 1 min in autoclaved seawater, and immersed in 1% Betadine solution with 2% Triton X-100 for 1 min to eliminate epiphytes. To liberate the monospores, the blades were cultured in Provasoli enriched seawater (PES) medium (Provasoli, 1968) under 40 μmol/m^2/s light intensity (10L:14D) at 20°C. Monospores were then grown to juvenile blades under the same conditions. Approximately 40 monospores (average size, 15 μm) were produced from an average 100 μm-long juvenile blade about every 20 days. Monospores were separated from juvenile blades by filtering with a 20-μm-mesh nylon membrane.

Seaweed extracts

Seaweed thalli for methanol and water extractions were collected from the coast of Korea from October 2002 to January 2005. The seaweed tissues were dried for 1 week at room temperature and then ground to a powder using a coffee grinder for 5 min. We added 1 L of methanol to each 20 g sample, and the methanol-soluble fraction was extracted at room temperature for 1 day; this was repeated 3 times, and the combined extracts were evaporated to dryness. We then added 1 L of distilled water to the seaweed tissue to extract the water-soluble components. A stock solution of each fraction was prepared by the addition of 1 mL of methanol or distilled water to each 40 mg of dried extract. The prepared stock solu-

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tions were diluted in seawater and filtered through a 0.22-μm filter before use.

Constituent separation and test compounds

The most active seaweed, *Sargassum sagamianum,* was fractionated by polarity into five classes of constituents: saccharides, lipids, phenolics, alkaloids, and nitrogen compounds (Harborne, 1998). The activity of ten known allelochemical and algicidal compounds, i.e., chitosan, cupric sulfate, di-n-octylphthalate, ferulic acid, salicylic acid, umbelliferone, Irgarol 1051, pyroglutamic acid, triethyl citrate, and tributyltin, were also tested for anti-attachment activity.

Anti-attachment activity

For the anti-attachment bioassay using the *P. yezoensis* monospores, 100 μL aliquots of seawater were distributed into wells cut from a 96 well plate. One μL of seaweed extract (40 mg/mL), 4 μL PES stock, and approximately 200 spores were added, and then the final volume was brought up to 200 μL immediately. The resulting spore suspensions were placed in the dark for 1 d at 20 °C to allow for even attachment to the bottom. At the end of this period, each well was placed in an inverted position into a 15 mL Falcon tube containing 1 mL seawater. Non-attached spores were removed from the well bottom by centrifugation at 1,500×*g* for 15 min, which pulled non-attached spores from the bottom substratum (Wagner et al., 1992). The number of attached spores remaining on the bottom was counted under an inverted microscope (×100) after replacing the PES medium. Spore attachment was expressed as a percentage of the value against the reference, which was prepared using the same procedure as above but without the seaweed extract or test compound. The minimum detectable inhibition of spore attachment by methanol occurred at 0.5% (data not shown). Therefore, the final concentration of methanol was kept below 0.5% in all tests.

Statistical analysis

The experiments were repeated at least three times for each independent assay. The means of each of the indicators were compared to the controls using student's *t*-test.

Results and Discussion

To establish an anti-attachment bioassay method using *P. yezoensis* monospores, we first examined attachment conditions for the monospores using centrifugation for the assay. Approximately 200 monospores were allowed to settle (sink and contact the bottom substratum) for 1 hr in wells cut from a 96-well plate. The spores that settled on the bottom were counted. They were then left to attach (by secreting complex adhesives) to the bottom in the dark at 20°C. The non-attached spores were removed from the bottom by centrifugation in an inverted position. The relative rate of attached to settled spores reached a stationary phase within 24 hr (Fig. 1). Another attachment assay using the rinsing method of Apple and Harlin (1995) was compared using the same monospores (data not shown). It attained the stationnary attachment phase within 3 days, and the rinsing strength was not well controlled. Since monospores and their germlings have a red pigment, the state of spore germination, the cell division of the germlings, and the production of rhizoids can be easily observed under a microscope. However, it takes at least 1 week to observe developmental changes. Thus, an assay for monospore attachment that could be determined using centrifugation after a 24 hr incubation in the dark at 20°C would be the quickest and most convenient method.

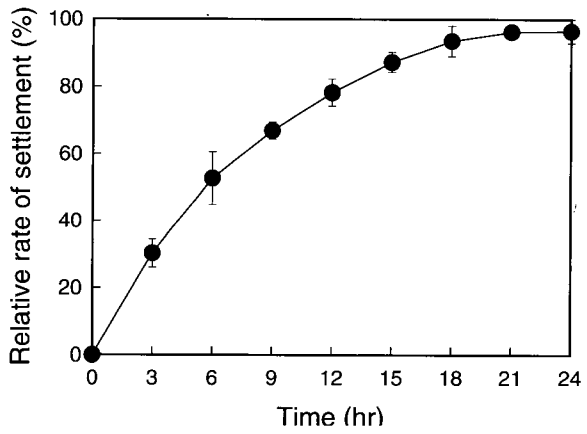

Fig. 1. Attachment rate of *Porphyra yezoensis* monospore in the dark at 20°C. The relative rate (%) is expressed as the number of attached spores over the number of settled spores.

The anti-attachment activities of 32 seaweed extracts were screened to detect allelochemical or antifouling compounds. Methanol- and water-soluble fractions were isolated from each seaweed to confirm the activity. The addition to the monospore suspensions of 200 μg/mL each of methanol extracts from *Monostroma nitidum, Ulva pertusa, Hizikia fusiformis, Ishige sinicola, Myelophycus simplex, Sargassum horneri, S. sagamianum, Scytosiphon lomentaria,* and *Corallina pilulifera* caused all spores to burst (Table 1). The methanol extracts from *En-*

Table 1. Effects of seaweed extracts on the attachment of *Porphyra yezoensis* monospores. Relative activity (%) = mean ± SD (n > 3). ND; not determined. [a]P<0.0001 and [b]P<0.001 compared to control.

	MeOH extract (μg/mL)		Water extract (μg/mL)	
	200	20	200	20
Chlorophyta				
Codium fragile	37±6[a]	ND	89±5	ND
Enteromorpha linza	1±1[a]	ND	87±6	ND
Monostroma nitidum	Burst	12±1[a]	54±4[a]	ND
Ulva pertusa	Burst	17±2[a]	72±4[b]	ND
Phaeophyta				
Colpomenia bullosa	86±13	ND	58±7[b]	ND
Colpomenia sinuosa	4±4[a]	ND	62±6[b]	ND
Ecklonia cava	59±5[b]	ND	61±10	ND
Endarachne binghamiae	4±3[a]	ND	96±6	ND
Hizikia fusiformis	Burst	70±9	97±5	ND
Ishige sinicola	Burst	6±2[a]	80±4[b]	ND
Kjellmaniella crassifolia	67±11	ND	76±3[b]	ND
Laminaria japonica	54±9b	ND	65±4[a]	ND
Myelophycus simplex	Burst	32±6	82±6	ND
Sargassum confusum	67±13	ND	90±3	ND
Sargassum horneri	Burst	7±3[a]	92±11	ND
Sargassum sagamianum	Burst	2±2[a]	97±9	ND
Sargassum thunbergil	81±7	ND	97±3	ND
Scytosiphon lomentaria	Burst	45±3[a]	95±7	ND
Undaria pinnatifida	1±1[a]	ND	80±7	ND
Rhodophyta				
Carpopeltis affinis	42±5[a]	ND	95±6	ND
Chondrus ocellatus	102±2	ND	88±7	ND
Corallina pilulifera	Burst	3±1[a]	75±5[b]	ND
Gigartina intermedia	1±1[a]	ND	91±3	ND
Gracilaria prolongata	13±4[a]	ND	90±2	ND
Grateloupia prolongata	49±15	ND	77±8	ND
Grateloupia turuturu	52±1[a]	ND	65±6[b]	ND
Hypnea charoides	1±1[a]	ND	93±6	ND
Laurencia pinnata	7±1[a]	ND	32±9[b]	ND
Lomentaria catenata	1±1[a]	ND	85±12	ND
Pachymeniopsis elliptica	0±0[a]	ND	Burst	34±11[b]
Porphyra yezoensis	4±2[a]	ND	55±15	ND
Symphyocladia latiuscula	100±4	ND	87±8	ND

teromorpha linza, *Colpomenia sinuosa*, *Endarachne binghamiae*, *Undaria pinnatifida*, *Gigartina intermedia*, *Hypnea charoides*, *Laurencia pinnata*, *Lomentaria catenata*, *Pachymeniopsis elliptica*, and *Porphyra yezoensis* itself showed significant attachment inhibition (>90%) compared to the reference. Adding the water extract from *P. elliptica* to the monospore suspension caused all spores to burst. None of the methanol or aqueous seaweed extracts enhanced attachment. Methanol extracts (20 μg/mL) from *I. sinicola*, *S. horneri*, *S. sagamianum*, and *C. pilulifera* showed significant attachment inhibition (>90%) compared to the reference. This activity was confirmed using 2 μg/mL of each extract. The methanol extract of *S. sagamianum* was the most active inhibitor of monospore attachment and was used for constituent separation.

Some allelochemicals have been reported from coralline algae. Compounds from *Lithophyllum* species destroy brown algal zoospores (Suzuki et al., 1998) and prevent the settlement and germination of the spores of several seaweed species (Kim et al., 2004). *Corallina pilulifera* showed strong algicidal activity against several red tide microalgae (Jeong et al., 2000). In our study, *C. pilulifera* also displayed strong anti-attachment activity against *P. yezoensis* monospores.

For constituent separation, *S. sagamianum* powder (100 g) was extracted with 10 L of methanol-water (4:1) three times, and the crude extract was evaporated, yielding a dark brown gummy residue (23 g). The extract was then successively fractionated according to polarity into five constituent classes: saccharides, lipids, phenolics, alkaloids, and nitrogen compounds (Table 2). The fraction that was acidified to pH 2 and extracted with chloroform yielded a moderately polar extract of phenolic compounds (3.2 g), which contained the main anti-attachment activity.

The response of *P. yezoensis* monospores to known allelochemical and algicidal compounds also showed good sensitivity (Table 3). We used a dose-response curve to determine the concentration resulting in 50% inhibition (IC_{50}) and the minimum concentration resulting in 100% inhibition (MIC). For chitosan, cupric sulfate, and Irgarol 1051, typical gradients of anti-attachment activity ranged logarithmically with their concentrations. Other test compounds inhibited attachment proportionately to their concentrations. Irgarol 1051, a commercial antifouling agent, was the strongest anti-attachment agent, with IC_{50} and MIC at nanomolar concentrations (Table 3). The treatments with tributyltin, cupric sulfate, and salicylic acid, which are all well-known antifouling or allelopathic agents, were also highly inhibitory at micromolar concentrations (Table 3).

We conducted a range of tests, including those of crude extracts, fractionated constituents, and known chemical compounds, to assess the possible inhibitory activities of various chemicals to monospore attachment. Our laboratory bioassay provides a valuable early indication of their allelopathic or antifouling action efficacy prior to field testing. Allelopathic plants or algae maintain or extend their territories by excreting allelochemicals that inhibit the growth of other species. In agriculture, allelochemicals that suppress or eliminate competing plants have received much attention due to their potential as selective natural herbicides (Vyvyan, 2002). The importance of allelochemicals that affect the behavior and distri-

Table 2. Comparison of the relative activity of different constituent fractions from *Sargassum sagamianum* on the attachment of *Porphyra yezoensis* monospore. Relative activity (%) = mean±SD (n>3). ND; not determined. [a]P<0.0001 and [b]P<0.001 compared to control.

	Saccharides	Lipids	Phenolics	Alkaloids	Nitrogen compounds
200 μg/mL	95±3	63±6	Burst	Burst	91±7
20 μg/mL	ND	ND	Burst	47±8[b]	ND
2 μg/mL	ND	ND	10±2[a]	ND	ND

Table 3. Comparison of the anti-attachment activity of known allelochemical and algicidal compounds on the attachment of *Porphyra yezoensis* monospores. The anti-attachment activity of IC_{50} and MIC was determined from dose-response curves.

Compounds	Unit	IC_{50}	MIC
Chitosan	%	0.1	5.1
Cupric sulfate	M	72.1	180.2
di-n-octylphthalate	mM	1.1	2.6
Ferulic acid	mM	3.0	6.1
Irgarol 1051	nM	13.4	513.0
Pyroglutamic acid	mM	163.3	407.0
Salicylic acid	M	133.3	261.5
Tributyltin	M	8.9	19.0
Triethylcitrate	mM	28.7	40.9
Umbelliferone	mM	3.6	6.9

bution of marine organisms has also been emphasized (Lobban and Harrison, 1994). The isolation of allelopathic compounds may lead to the discovery of new antifouling agents based on selective natural products from the marine environment. *Enteromorpha, Sargassum, Ulva* and other marine algae have been used as assay organisms for screening specific allelopathic or antifouling agents (Fletcher, 1989; Hellio et al., 2002). However, spore liberation from these seaweeds has a marked tidal periodicity. Most spores are liberated during the highest tides in each lunar period. In addition, spore liberation decreases or disappears depending on habitat and season (Shin and Smith, 1996). In contrast, *P. yezoensis* monospores are easily obtainable throughout the year by small-scale cultivation. Thus, our anti-attachment bioassay using *P. yezoensis* monospores is a promising screening method for allelopathic seaweeds or antifouling compounds in the marine environment.

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