Isolation of algal spore lytic C17 fatty acid from the crustose coralline seaweed *Lithophyllum yessoense*

Quoc-Hai Luyen · Ji-Young Cho · Jae-Suk Choi · Ji-Young Kang · Nam Gyu Park · Yong-Ki Hong

Abstract The algal spore lytic fatty acid of heptadeca-5,8,11-trien (HpDTE: C17:3) was isolated from the crustose coralline seaweed *Lithophyllum yessoense*. HpDTE, an odd-numbered carbon fatty acid, showed more than 50% lysis at a concentration of 5 $\mu g.mL^{-1}$ against the spores of three chlorophyte species, nine rhodophytes, four phaeophytes, and the cells of four phytoplankton species. Lysis activity increased with the number of double bonds and carbon atoms in the fatty acid increased. HpDTE showed a ten-fold stronger activity with a LC_{50} of 3.1 $\mu g.mL^{-1}$ than α-linolenic acid (C18:3).

Keywords Algal spore lysis · C17 fatty acid · Crustose coralline · Heptadeca-5,8,11-trien · *Lithophyllum yessoense*

Introduction

Many areas of the rocky shorelines of Korea and Japan are currently dominated by crustose coralline algae such as *Lithophyllum yessoense* Foslie (Suzuki et al. 1998; Kim 2000). These non-articulated (non-geniculate) calcareous

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algae cover the surfaces of rocks in a pink or white-colored crust. The decrease in the seaweed flora of some rocky areas, known as alga whitening or barren ground, is associated with some species of crustose algae (Tokuda et al. 1994).

Even though competitive allelopathy between crustose coralline algae and fleshy seaweeds has received little attention, coralline algae produce some allelopathic substances. Suzuki et al. (1998) isolated an allelopathic substance active against zoospores of the brown seaweed *Laminaria religiosa* from the crustose coralline alga *Lithophyllum* sp., but did not determine its structure. Kim et al. (2004) investigated the possible formation of multiple allelopathy-related substances by *L. yessoense* against the settlement and germination of spores from 17 species in 15 genera of seaweed. Kakisawa et al. (1988) isolated an allelopathic substance from the brown alga *Cladosiphon okamuranus* and identified it as octadeca-6,9,12,15-tetraenoic acid (C18:4) against 37 species of microalgae. Suzuki et al. (1996) isolated an allelopathic substance from the red alga *Neodilsea yendoana* and identified it as eicosapentaenoic acid (C20:5). Chiang et al. (2004) reported that the fatty acids α-linolenic acid (C18:3), oleic acid (C18:1), linoleic acid (C18:2), and palmitic acid (C16:0) of the green alga *Botryococcus braunii* showed allelopathic activity against a variety of phytoplankton and zooplankton. Alamsjah et al. (2005, 2008) reported the algicidal substances hexadeca-4,7,10,13-tetraenoic acid (C16:4), octadeca-6,9,12,15-tetraenoic acid, α-linolenic acid, and linoleic acid derived from *Ulva fasciata*.

Here we describe the isolation and identification of the odd-numbered carbon atom C17 fatty acid from *L. yessoense* and its lytic activity against several types of seaweed spores, and compare its activity to that of other fatty acids (C16–C22).

Materials and methods

Pink crustose coralline tissues of *Lithophyllum yessoense* Foslie were collected from rocks in the lower intertidal zone of the shore at Chungsapo (35°09′28″ N, 129°11′47″ E), on the east coast of Busan, Korea. Stones covered with healthy algal tissue were transported in a seawater tank to the laboratory on the same day. White or grayish pink patches were removed from the crustose thalli using a grinding drill and steel saw. After rinsing well with autoclaved seawater to remove potential contaminants, the tissues were brushed and cleaned three times with 30-s pulses of an ultrasonic water bath (low-intensity frequency of 90 kc s^{-1}) to eliminate micro-epiphytes. After sonication, the samples were dried completely for 2–3 days at room temperature using a fan.

Isolation of lytic compound

Approximately 12 kg of dried *L. yessoense* tissues were extracted with 50 L solution of methanol-water (4:1) at room temperature for 1 day, and then filtered through a Whatman GF/C filter under reduced pressure. This extraction procedure was repeated three times, and the extracts were combined. After filtration, the crude extract was concentrated to give a dark green residue (206 g) under reduced pressure in an evaporator. The extract was successively fractionated into different classes according to polarity following Harborne (1998). The fraction that was acidified to pH 2 using sulfuric acid and extracted three times with chloroform, resulted in a moderately polar extract that contained the main lytic activity. This fraction was loaded on a silica gel column (5×80 cm; 70–230 mesh) and eluted sequentially with 500 mL of hexane:diethyl ether (8:2), diethyl ether, acetone, and EtOAc:MeOH (8:2). The acetone eluent (4.14 g) with activity was dried and dissolved in MeOH, then further fractionated on a Sephadex LH-20 (Pharmacia) column (2.5 cm×100 cm) using 100% MeOH as the eluent. Each 2-mL fraction was collected at a flow rate of 0.5 mL min^{-1}. The active substance was obtained from the fractions 80–100 (1.01 g), which were collected, dried, and dissolved in 3 mL MeOH for reverse-phase high performance liquid chromatography (RP-HPLC). Separation of each 150 μL was achieved using an Ultraspere C18 column (10 mm ID×25 cm). The analysis was performed on a Waters 600 gradient liquid chromatograph monitored at 202 nm. The mobile phase consisted of two solvent systems: acetonitrile and water. Elution was performed with a linear gradient of 0–100% acetonitrile for 60 min at a flow rate of 2 mL min^{-1} to yield the pure compound (2.7 mg).

Analytical methods

The purified compound was analyzed on a JEOL JNM-ECP 400 NMR spectrometer (Tokyo, Japan), operating at 500 and 100 MHz for ^{1}H and ^{13}C, respectively, using methanol-d (CD$_3$OD). Infrared spectrum was recorded on a Fourier Transform IR spectrophotometer (IFS-88; Brucker, Karlsruhe, Germany). The structure of the purified compound was referred by comparing it to the structure of a C17 carbon fatty acid from Saito and Ochiai (1996).

Bioassay of algal spore lysis

The isolation procedure of the active compound was monitored using a lysis assay with monospores of *Porphyra suborbiculata* Kjellman. Juvenile blades were collected from the intertidal zone of the rocky shore at Chungsapo, Busan, Korea. Axenic isolation and culturing of the monospores followed Choi et al. (2002, 2005). For spore collection from various fleshy seaweeds, fertile thalli of 16 different seaweed species were collected from the coast of Korea. The thalli were cleaned, dried, and induced to release spores using sterilized seawater (Kim et al. 2004). Microalgal cells were obtained from the Korea Marine Microalgae Culture Center.

Various amounts of the purified compound in MeOH (2 μL) were added to 198 μL Provasoli's enriched seawater (PES) medium (Provasoli 1968) containing approximately 200 spores or cells, and then incubated under 40 μmol photons m^{-2} s^{-1} light at 20°C for 4 h. The number of spores remaining was counted under a microscope (×100). Spore lysis (%) was expressed as a relative rate: $[(C–S)/C]×100$, where C is spore number in controls (without the compound) and S is spore number of samples (with the compound). Assays for anti-attachment and anti-germination followed Kim et al. (2004). The minimum detectable lysis, anti-attachment, and anti-germination activities of spores by MeOH occurred at 1% (data not shown). Therefore, the final concentration of MeOH was kept below 1% in all tests.

Statistics

Each independent assay was repeated at least three times with separate cultures. Treatment means were compared to controls using Student's *t*-test.

Results

The active compound was eluted at 100% (in 70.9 min) acetonitrile by RP-HPLC. It was a colorless oily compound,

weighing 2.7 mg, and yielding $2.3 \times 10^{-5}\%$ or $1.3 \times 10^{-3}\%$ from the dried seaweed tissue or the crude extract, respectively. Infrared (dry film) analysis of the purified compound showed absorptions for OH (3400–3000 cm^{-1}) and carbonyl function (1708 cm^{-1}). The ^{1}H NMR spectrum revealed a methyl proton at δ 0.88 (3H, t, H-17), nine methylene protons at [δ 1.33–1.29 (6H, m, H-14, H-15, H-16), δ 1.64 (2H, m, H-3), δ 2.05 (2H, m, H-13), δ 2.11 (2H, m, H-4), δ 2.26 (2H, t, H-2), δ 2.84–2.81 (4H, m, H-7, H-10)], and six methine proton signals at δ 5.38–5.33 (6H, m, H-5, H-6, H-8, H-9, H-11, H-12); the proton of the carboxyl group (H-1) was at δ 3.33. The ^{13}C NMR spectrum revealed one carbonyl carbon at δ_C 177.8 (C-1), one methyl carbon at δ_C 14.43 (C-17), nine methylene carbons [δ_C 23.64 (C-16), δ_C 26.11 (C-3), δ_C 26.55 (C-7, C10), δ_C 27.63 (C-4), δ_C 28.19 (C-13), δ_C 30.47 (C-14), δ_C 32.67 (C-15), δ_C 34.62 (C-2)], and six methine carbons [δ_C 128.78 (C-11), δ_C 129.11 (C-6), δ_C 129.43 (C-9), δ_C 129.81 (C-8), δ_C 130.13 (C-5), δ_C 131.18 (C-12)]. Assignments were made by analyzing the HMQC, HMBC, and COSY spectra. From the COSY spectrum, we determined the first double-bond position from the terminal methyl carbon (C-17). The terminal methyl proton H-17, the methylene protons H-16, H-15, H-14, and H-13, and the methine proton H-12 showed a series of COSY correlations with each other, demonstrating that the first double bond from the terminal methyl carbon (C-17) was at the sixth position (n-6 or C-12). From these spectral data, we identified the compound as the polyunsaturated fatty acid heptadeca-5,8,11-trien (C17:3 n-6; Fig. 1).

Spore lytic activity of the isolated HpDTE

When the isolated HpDTE was added to PES medium containing monospores of *P. suborbiculata*, a lysis reaction occurred quickly and reached maximum within 4 h at each concentration tested (Fig. 2).

Lysis activity on the spores of 16 seaweed species and the vegetative cells of four microalgae was measured (Table 1). In general, HpDTE showed significant lytic activity against most spores and cells. Adding 25 µg.mL^{-1}
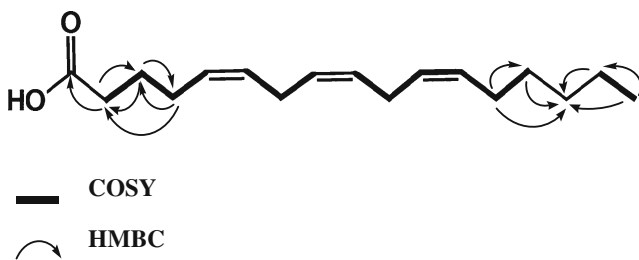

Fig. 1 Structure of heptadeca-5,8,11-trien (C17:3 n-6) isolated from the crustose coralline seaweed *Lithophyllum yessoense* Foslie
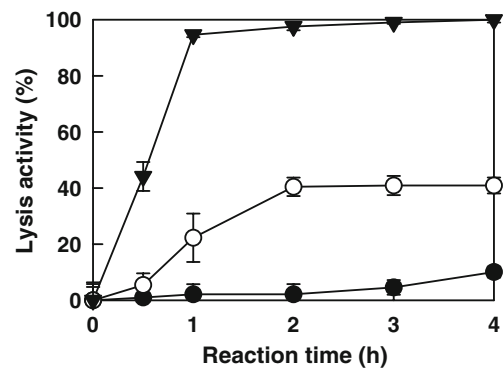

Fig. 2 Lysis activity of the isolated HpDTE on monospores of *P. suborbiculata*. Concentrations of 1 µg/mL (●), 2.5 µg.mL^{-1} (○), and 5 µg.mL^{-1} (▾) of HpDTE were added to PES (200 µL) containing approximately 200 monospores. Values represent the mean ± SD ($n \geq 3$)

HpDTE to spores, especially those of *Lomentaria catenata* and *Dictyota dichotoma*, did not cause the spores to burst. The microalga *Alexandrium catenella* was also little affected at this concentration. These data imply that *L.*

Table 1 Lysis activity of the purified HpDTE against seaweed spores and microalgal cells

	Concentration of HpDTE (µg mL^{-1})		
	25	5	1
CHLOROPHYTA			
Enteromorpha compressa	100±0*	100±0*	76±3*
Enteromorpha linza	100±0*	100±0*	72±2*
Ulva pertusa	92±2*	51±3*	28±5
RHODOPHYTA			
Carpopeltis affinis	90±2*	68±2*	13±3
Carpopeltis prolifera	100±0*	84±2*	2±4
Chondrus ocellatus	94±1*	74±4*	14±2
Gracilaria verrucosa	100±0*	100±0*	6±3
Gymnogongrus flabelliformis	100±0*	68±5*	2±4
Lomentaria catenata	16±8	8±3	0±3
Porphyra suborbiculata	100±0*	97±1*	10±2
Porphyra yezoensis	100±0*	96±2*	14±9
Symphyocladia latiuscula	100±0*	71±2*	14±3
PHAEOPHYTA			
Dictyota dichotoma	22±5	18±9	5±9
Laminaria japonica	90±2*	71±5*	1±5
Sargassum horneri	100±0*	87±3*	44±6
Undaria pinnatifida	88±3*	66±3*	2±4
MICROALGAE			
Alexandrium catenella	32±9	7±9	6±6
Chattonella sp. RA005	100±0*	100±0*	77±5
Cochlodinium polykrikoides	100±0*	90±2*	16±7
Heterosigma akashiwo	100±0*	100±0*	89±6*

Each concentration of HpDTE (µg mL^{-1}) was added to PES with spores or cells for 4 h. The activity is expressed as the relative rate of spore lysis (%). Values represent the mean ± SD ($n \geq 3$). Statistical significance is *$p < 0.001$ as compared to controls

Table 2 Lysis activity of C16–C22 fatty acids on monospores of *P. suborbiculata*

Fatty acids	LC_{50} (μg/mL)
Palmitic acid (C16:0)	>150
Oleic acid (C18:1 n-9)	>100
Linoleic acid (C18:2 n-6)	40.1
α-Linolenic acid (C18:3 n-3)	35.4
HpDTE (C17:3 n-6)	3.1
Arachidonic acid (C20:4 n-6)	1.8
Eicosapentaenoic acid (C20:5 n-3)	2.1
Docosahexaenoic acid (C22:6 n-3)	0.8

The LC_{50} was determined from dose-response curves

yessoense produces a lytic compound against spore settlement of most, if not all, seaweeds that occur on crustose coralline algae flats, and thereby decreases the seaweed flora in such areas. Because the spores of *L. catenata* and *D. dichotoma* showed low levels of lysis, restoration of algal whitening areas might be possible by first transplanting these species, and later adding other seaweeds.

HpDTE at a concentration of 0.5 μg mL^{-1}, which showed no lysis activity, was tested for inhibitory activity against settlement and germination of seaweed spores. The compound showed almost no effects on spore settlement of most seaweed species, although it reduced spore settlement to less than 80% of controls in *D. dichotoma*, *Gymnogongrus flabelliformis, L. catenata*, and *Ulva pertusa*. There was no substantial inhibition of spore germination by HpDTE (data not shown).

Spore lytic activity of eight different C16–C22 fatty acids was measured on monospores of *P. yezoensis*. Using a dose-response curve, we determined the concentration resulting in 50% lysis (LC_{50}). HpDTE showed an LC_{50} value of 3.1 μg/ mL (Table 2). Fatty acids with a higher degree of unsaturation in the carbon chain exhibited higher activity. Although α-linolenic acid (n-3) and HpDTE (n-6) had the same level of unsaturation, the LC_{50} value of HpDTE was ten times higher. As the number of carbon atoms with unsaturation increased, lysis activity increased. Docosahexaenoic acid, which had the highest number of unsaturated carbons, showed the strongest LC_{50} (0.8 μg/mL).

Discussion

In areas with algal whitening there are commonly no fleshy seaweed epiphytes on the pink crustose coralline surfaces. Although causes such as herbivore induction (Kitamura et al. 1993), grazing (Agateuma et al. 1997), physical sloughing (Masaki et al. 1984; Johnson and Mann 1986), and iron deficiency (Suzuki et al. 1995) may be sufficient to prevent recruitment of fleshy seaweeds, allelopathic substances may also destroy (Suzuki et al. 1998) and/or help prevent the settlement or germination of seaweed spores (Kim et al. 2004).

We isolated a novel polyunsaturated fatty acid (PUFA) of HpDTE (C17:3) that shows potent lysis activity against seaweed spores. Odd-numbered carbon atom fatty acids are typically toxic (Mackay et al. 1940), and we found that HpDTE showed more potent lysis against seaweed spores than α–linolenic acid (C18:3). In general, PUFAs, whether free acids, phospholipids, or glycolipids, are usually found in algal extracts and are considered components of the membrane. The composition of PUFAs can be used as an indicator of food quality (Wood et al. 1999). However, high concentrations of PUFAs may be toxic (Yasumoto et al. 1990). For example, EPA (C20:5 n-3) exhibits lethal toxicity to various microalgae and macroalgae. We isolated EPA also from *L. yessoense* that lyses seaweed spores, although the extractable amount was lower than that of HpDTE. Jüttner (2001) proposed that the PUFAs observed in algal extracts are in fact the first products of the lipoxygenase cascade that starts upon cell disruption. Fu et al. (2004) assumed that PUFAs could be part of a defense strategy that rapidly converts an essential cell constituent into highly toxic compounds against grazers. Unsaturated fatty acids produce free radicals when oxidized in seawater, and they may attach to algae as toxins (Murata et al. 1989). Thus, marine plants might commonly use PUFAs as allelochemicals to inhibit the growth of competing macro- or microalgae. The high toxicity of HpDTE may also be due to the amphiphatic properties of PUFAs, which probably disrupt the membrane integrity of seaweed spores and microalgal cells. Chemical structural features such as the number of unsaturated double bonds may also be involved in biological activity (Chiang et al. 2004).

Even though *L. yessoense* produces a spore lytic compound against spore settlement of most seaweeds, little lysis occurred in the spores of *L. catenata* and *D. dichotoma*. No inhibition of settlement or germination occurred in spores of *D. dichotoma* (Kim et al. 2004). Thus the restoration of algal whitening areas might be possible by transplanting these species first, and then inducing the recruitment of other seaweeds.

In conclusion, we isolated an odd-numbered carbon atom fatty acid of HpDTE from the crustose coralline *L. yessoense* as a spore lytic compound against diverse seaweeds and microalgae. Our results support claims that crustose coralline algae may use PUFAs as a chemical defense mechanism against competing algae.

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