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Growth inhibition of unicellular and colonial *Microcystis* strains (*Cyanophyceae*) by compounds isolated from rice (*Oryza sativa*) hulls

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ABSTRACT

The algicidal effects of crude and pure rice hull extracts on the growth of *Microcystis aeruginosa* were investigated using cultured unicellular and colonial strains. Upon treatment with rice hull crude extract (RHE), growth inhibition of unicellular *M. aeruginosa* was much higher than that of colonial *M. aeruginosa*. However, purified compounds from the crude extract, β-sitosterol-β-D-glucoside and dicyclohexanyl orizane, powerfully inhibited the growth of colonial *M. aeruginosa* cells. At the same concentrations, the two compounds were almost equipotent (66% and 80% growth inhibition for colonial *M. aeruginosa*, respectively; $P < 0.05$). As rice hulls are readily obtainable, and as extracts show high algicidal activity (targeting colonial algae rather than unicellular organisms) at low concentrations, the results suggest that some pure compounds extracted from rice hulls, such as β-sitosterol-β-D-glucoside and dicyclohexanyl orizane, may serve as environmentally friendly agents for controlling the growth of toxic colonial *M. aeruginosa* in eutrophic waters.

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1. Introduction

Cyanobacterial blooms in eutrophic freshwater ecosystems are a major problem worldwide, causing deterioration of water quality, illness in wildlife, livestock, and humans, and mortality in dogs and cattle (Carmichael, 1994; Christoffersen, 1996). *Microcystis aeruginosa* is the most common species of bloom-causing cyanobacteria. Many strains of *Microcystis* are known to produce cyanobacterial hepatotoxins termed microcystins (Oh et al., 2000; Yasuno et al., 2000). These toxins, which are soluble peptides, damage the livers of higher animals (Codd and Poon, 1988; Watanabe et al., 1989) and are lethal or harmful to many kinds of aquatic organisms (Penaloza et al., 1990). Therefore, control of microcystin-producing *Microcystis* is an important environmental and public health issue.

Various chemical or synthetic agents (e.g., copper, chlorine, aluminum, calcium, and potassium permanganate) are used to control nuisance phytoplankton and weeds in aquatic ecosystems. However, these algicides often induce secondary pollution such as the release of phytotoxins, which threaten drinking water supplies, persist in the environment, and are toxic to fish (Lam et al., 1995; Karan et al., 1998; Boyd and Massaut, 1999; Meepagala et al., 2005).

In recent efforts to control toxic bloom-forming *Microcystis*, algicides from natural biomaterials have received attention as alternatives to chemical agents. Such algicides are likely to be specific and biodegradable, and may therefore offer an environmentally friendly method for control of algal blooms (Park et al., 2006a,b) when prevention is not an option. Nakai et al. (2000) showed that allelopathic compounds from the macrophyte *Myriophyllum spicatum* inhibited the growth of *M. aeruginosa*, and Hehmann et al. (2002) reported that L-lysine, lysine malonate, and lysine copper specifically inhibited the growth of *Microcystis* species. Also, Kodani et al. (2002) found that 1-methyl-β-carboline, produced by the bacterium *Pseudomonas* sp. K44-1, was active against bloom-forming cyanobacteria such as *M. aeruginosa* and *M. viridis*. Furthermore, the growth of *M. aeruginosa* is inhibited by L-2-azetidinecarboxylic acid from *Polygonatum odoratum* var. *pluriflorum* (Kim et al., 2006), salicylic acid from rice straw (Park et al., 2006a), tannic acid from oak extracts (Park et al., 2006b), and several compounds isolated from rice hulls (Chung et al., 2007). However, these studies used unicellular *M. aeruginosa* strains. We think that it is important to test the effects of natural algicides on problematic colonial *M. aeruginosa*. Extracts from rice hulls have recently been found to have herbicidal and algicidal effects (Chung et al., 2007). Rice is the principal cereal in Asian countries and the staple food of over half the world's population. In Korea, rice is currently produced at an annual level of approximately 4.5 million tonnes (data from Korea National Statistical Office, http://www.kosis.kr/index.html). Rice hull is the major by-product of

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milling and represents approximately 20% of rough grain weight (Xuan et al., 2003). Thus, rice hulls, which are otherwise an agricultural waste product, may be an environmentally friendly and sustainable source of a bioalgicide.

In the present study, we investigated the effect of rice hull extracts (crude extract and purified compounds) on the growth of both unicellular and colonial *M. aeruginosa* strains, to determine if the hulls might contain environmentally friendly algicides, with particular attention to growth inhibition of a naturally colonizing strain.

2. Materials and methods

2.1. Unicellular and colonial Microcystis strains

The unicellular *M. aeruginosa* strain NIER 10010 was obtained from the National Institute of Environmental Research, Korea. The colonial *M. aeruginosa* was isolated from a eutrophic lake (Lake Ilgam, Seoul, Korea) and identified to the species level following the criteria of Akiyama et al. (1981), under a light microscope (Olympus, Tokyo, Japan). The unicellular strain NIER 10010 has been reported to produce microcystin (Lee et al., 2007), and microcystin production by the colonial strain was also detected by ELISA (unpublished data).

The unicellular strain was grown in CB medium, pH 9.0 (Shirai et al., 1989) and the colonial strain was grown in Allen medium, pH 7.8 (Allen, 1968). All strains were maintained for 2 weeks at 25 °C on a 14 h:10 h light/dark cycle in a shaking incubator with illumination at 100 μmol photons m^{-2} s^{-1} provided by cool-white fluorescent lamps. For seed cultures, 10 mL of culture was transferred into a 250 mL culture flask with 90 mL of fresh medium.

2.2. Rice plant material

An *Oryza sativa* cultivar (Ilpumbyeo) was grown in an experimental field at Konkuk University, Korea, and harvested in October 2002. A voucher specimen of hulls (reference code KKU 121, Ilpumbyeo) has been deposited in the herbarium of the Department of Applied Life Science, Konkuk University. Rice hulls from harvested plants were separated with a milling machine and dried at room temperature (25 °C) for 7 days. The dried rice hulls were pulverized using a Wiley mill and passed through a 40-mesh screen.

2.3. Preparation of rice hull crude extract (RHE)

The powdered rice hulls (10 kg) were immersed in methanol (60 L) for one week at room temperature, and the supernatant was concentrated under vacuum to yield 150 g of crude extract. The dried extract was resuspended in water (1 L) and extracted with ethyl acetate (EtOAc) three times (each 500 mL) to produce 35 g of material. The ethyl acetate extract was stored in a freezer (−20 °C) until use.

2.4. Fractionation of RHE

The dried ethyl acetate extract (35 g) was mixed with silica gel (60–120 mesh size) and subjected to normal-phase column chromatography over silica gel in a glass column eluted with the following solvents: fraction 1, hexane; fractions 2–5, hexane:ethyl acetate (9:1); fractions 6–11, hexane:ethyl acetate (8:2); fractions 12–15, hexane:ethyl acetate (7:3); fractions 16–20, hexane:ethyl acetate (1:1); fractions 21–22, ethyl acetate; fractions 23–28, ethyl acetate:methanol (9.5:0.5); fractions 29–32, ethyl acetate:methanol (9:1); fractions 33–34, ethyl acetate:methanol (7:3); fractions 35–40, methanol.

2.5. Isolation of compounds from chromatography fractions

Fractions 1 and 5 were separated by further chromatography and thin-layer chromatography over silica gel in hexane and hexane:ethyl acetate (9:1) to yield two pure compounds: hentriacontane (100 mg) and 1-tetratriacontanol (50 mg). Fraction 6 was crystallized and, after purification by column chromatography, yielded β-sitosterol (1 g) as confirmed by comparison with pure β-sitosterol (Sigma, St. Louis, MO). Fraction 9 was further purified by column chromatography over silica gel using methylene chloride, and then methylene chloride and methanol in different ratios, to yield orizaterpenoid. Column chromatography of fractions 10–11 yielded stearic acid and myristic acid. Because of insolubility in most solvents, stigmast-5-en-3α-26-diacetate, obtained from fractions 16–20, was prepared as the acetylated product. Fraction 23, after column chromatography over silica gel and elution with chloroform and methanol, yielded two pure compounds, dicyclohexanyl orizane and β-sitosterol-β-D-glucoside. The structures of nine relevant compounds are presented in Fig. 1.

2.6. Inhibition of algal growth by RHE and pure compounds

For all growth inhibition experiments, *M. aeruginosa* was cultured at 25 °C with illumination at 100 μmol photons m^{-2} s^{-1} with a 14 h:10 h light–dark photoperiod, in a culture room. For cell counting, 1 mL aliquots were taken from test flasks and fixed in Lugol's solution (APHA, 1995).

The effects of the RHE (1, 10, 100, and 1,000 μg L^{-1} on unicellular *M. aeruginosa*; 0.01, 1, 100, and 10,000 μg L^{-1} on colonial *M. aeruginosa*) and the pure compounds (100 and 1,000 μg L^{-1} on unicellular *M. aeruginosa*; 100 μg L^{-1} on colonial *M. aeruginosa*) were tested using *M. aeruginosa* cultivated in post-filtered (GF/C filter; pore size, ~0.7 μm) eutrophic lake water (Lake Ilgam, Seoul, Korea). Filtered lake water included considerable amounts of nitrogen and phosphorus, at concentrations of 2.05 mg L^{-1} (Total N) and 0.14 mg L^{-1} (Total P), respectively. Thus, we did not add additional nutrients during the test period. Although we suspected that the pore size of the GF/C filter might permit some natural bacteria to pass through, we nonetheless used natural lake water in tests to better reflect the field situation. Numbers of unicellular *M. aeruginosa* were counted using a hemocytometer (Fuchs-Rosenthal type; Paul Marienfeld GmbH & Co., Lauda-Königshofen, Germany) under a phase-contrast microscope (Axioplan, Zeiss, Oberkochen, Germany). Colonial *M. aeruginosa* was sonicated (JAC 4020, KODO Co., Seoul, Korea) for 60 s at 50 °C to separate cells before counting.

2.7. Data analysis

Algicidal activity was calculated using the following equation: Algicidal activity (%) = [(control cell number − cell number after treatment)/control cell number] × 100. The data were expressed as the means ± SEs of triplicate experiments. Differences in cell densities between treated and control cultures were analyzed by ANOVA with Duncan's multiple-range test (SPSS Inc., Chicago, IL; 2003 version). A *P*-value of less than 0.05 was considered to indicate a significant difference.

3. Results

3.1. Growth inhibition by RHE

A marked inhibitory effect of RHE on unicellular *M. aeruginosa* was noted (Fig. 2). At all four RHE concentrations (1, 10, 100, and 1,000 μg L^{-1}) tested, the numbers of unicellular *M. aeruginosa*

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