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Algicidal effects of free-amine water-soluble chitosan to marine harmful algal species

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ABSTRACT

Harmful algal blooms (HABs) commonly cause significant damage to aquatic systems due to toxin production and depletion of oxygen. In this study, the low molecular weight water-soluble chitosan (LMWSC) was investigated as a new algicidal compound by first investigating the potential of LMWSC in selective combating HABs. The LMWSCs disrupted algal cell and chloroplast membranes within 1 h against HABs, leading to efflux of the intracellular components. For co-cultivation of harmful and harmless algal, LMWSC selectively kill the harmful algal. Based on these results, LMWSC is a promising novel material for harmful algal bloom control.

Introduction

Harmful algal blooms (HABs) commonly cause mass mortalities of fish, shellfish, and other oceanic life due to toxin production and depletion of oxygen [1,2] Repeated and widespread HABs can drastically change marine ecologies. Furthermore, ingestion of seafood with algal toxins can lead to illness and death in humans and animals, and exposure to aerosolized toxins from contaminated water causes respiratory problems in humans [3,4]. Frequent occurrence of HABs has been interested in worldwide, likely due to several factors including climate change and environmental pollution [5,6]. In recently, there has been a focus on development of early warning systems and oceanographic tracking models to predict costal blooms and minimize losses to commercial fisheries or aquaculture [7,8]. However, the occurred HABs must be directly applied by anti-algal materials, controlling and preventing HABs.

For these reasons, it is important to develop materials and methods to prevent or control HABs in marine environments, aquaculture industry, and human health. Therefore, many researchers have endeavored to prevent HABs worldwide. However, approaches proposed to date such as treatment with fatty acids [9,10], algicidal bacteria [11–14], viruses [15], algicidal compounds [16–18], surfactants [19], and clay dispersion [20–22] have high cost and sometimes lead to secondary environmental pollution and alteration of the ecological system. Many scientists have reported some chemicals that mitigate HABs, but biological investigations of the safety of chemicals are needed due to their side effects and disturbance of the ecological system. Numerous investigations for use of algicidal bacteria have been conducted to control HABs [11,13,14]. But this method remains cytotoxic and ecological study for safety when algicidal bacteria are treated in high cell density.

Chitosan was recently used to convert sand into effective flocculants for mitigation of HABs in marine and freshwater systems [23] and soil/clay flocculation [24,25]. However, there have been no reports of the use of chitosan alone for removing and controlling HAB. Generally, anti-biological use of chitosan has been limited to high molecular weight and salt forms, because this has occasionally induced cytotoxicity and been difficult to control biological functions in physiological pH. To overcome these problems, we developed the novel salts-removal method which prepares low molecular weight water-soluble chitosan (LMWSC) with free amine groups [26].

In this study, we investigated the selective algicidal effect of LMWSCs with different molecular weight range against dinophyceae (*Alexandrium catanella*, *Alexandrium tamarense*, *Cochlodium polykrikoides*, *Gyrodinium impudicum*, *Prorocentrum micans*, and *Prorocentrum minimum*) and raphidophyceae (*Chattonella marina*

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and *Heterosigma akashiwo*) species, which induce harmful algal blooms (HAB) and the cytotoxicity of LMWSCs. Furthermore, we demonstrated that the LMWSC is a novel material with the potent algicidal activity.

Materials and methods

Materials

LMWSC (DDa (degree of deacetylation) > 98.0%, Mw: 1.4, 4.7, 12.7, and 22 kDa) and water in soluble chitosan (WISC, DDa > 98.0%, Mw; 22 kDa) were acquired from Kitolife Co. Ltd. (Seoul, Korea). SYTOX green and fluorescein isothiocyanate (FITC) were obtained from Molecular Probes (Eugene, OR). All chemicals used for preparation of f/2 medium were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

Algal cultures and algicidal activity

A. catanella, *A. tamarense*, *Chattonella* sp., *G. impudicum*, *H. akashiwo*, *P. micans*, *P. minimum* and *Skeletonema costatum* were provided by the Korea Marine Microalgae Culture Center, Busan, Republic of Korea. *C. polykrikoides*, *H. akashiwo* sp., and *C. marina* were collected from the coastal area of the South Sea in Korea. All species except *S. costatum* were grown and maintained in an f/2 medium [27] at 20 °C with 16/8 h light/dark illumination cycles under cool white fluorescent light (120 μmol photons m^{-2} s^{-1}). *S. costatum* was grown under the same conditions, but under a light density of 30 μmol photons m^{-2} s^{-1}.

Algal cultures at the mid-exponential phase were introduced into a 12-well tissue culture plate at a concentration of 1–2 × 10^4 cells/mL, and twofold serial dilutions of each LMWSC with f/2 medium were added. After incubation for 2 h (for raphidophyceae), 6 h (for dinophyceae), or 48 h (for *S. costatum*), the numbers of surviving cells were counted in a hemacytometer with a Sedgwick–Rafter counting chamber using an Olympus IX71 microscope equipped with a DP71 camera (Olympus, Tokyo, Japan). The immotile concentration (IC) or algicidal concentration (AC) of LMWSCs was defined by counting the number of non-swimming cells or burst cells in which cell envelopes or membranes were completely disrupted, respectively.

Pre-cultured *H. akashiwo* or *P. minimum* cells were adjusted to a concentration of 2.2 × 10^4 cells/mL and *S. costatum* cell cultures were adjusted to 2.3 × 10^4 cells/mL. After mixing cultures of *H. akashiwo* with *S. costatum* or *P. minimum* with *S. costatum*, the AC_{90} of LMWSC-3 was added and cells were counted at the indicated times in a hemacytometer with a Sedgwick–Rafter counting chamber.

Assay for chlorophyll a

To measure the chlorophyll *a* concentrations, *H. akashiwo* cells incubated in the absence or presence of chitosans (LMWSCs and WISC) were harvested by centrifugation (at 3000 × *g* for 10 min) at the indicated times. The cell pellets were then resuspended and extracted in 90% acetone for 24 h at 4 °C. Next, the samples were centrifuged at 10,000 × *g* for 10 min to remove cell debris, after which the chlorophyll *a* concentrations were determined as described by Jeffrey and Humphrey [27].

Hemolysis

Fresh fish blood was collected from *Sebastes schlegeli* obtained from fish farms in the South Sea of Korea and immediately injected into heparin blood collection tubes (BD Vacutainer, Franklin Lakes, NJ). After gentle mixing, red blood cells (RBCs) were centrifuged at 800 × *g* and washed with PBS until the supernatant was clear. The RBCs (8%, v/v of the final concentration) were added to chitosan that had been subjected to 2-fold serial dilution with PBS. After incubation with mild agitation for 1 h at 37 °C, the samples were centrifuged at 800 × *g* for 10 min and the absorbance of the supernatant was then measured at 414 nm. Complete (100%) or no hemolysis was defined as the absorbance of the RBCs containing 1% Triton X-100 or PBS alone, respectively. Each measurement was made in triplicate, and the percentage hemolysis was calculated using the following equation [18]:

$$\text{Hemolysis}\ (\%) = \frac{\text{Abs}_{\text{chitosan}} - \text{Abs}_{\text{PBS}}}{\text{Abs}_{\text{Triton-X100}} - \text{Abs}_{\text{PBS}}} \times 100 \quad (1)$$

SYTOX green uptake

Algal cells were grown to the mid-exponential phase under the above culture conditions and adjusted to 4 × 10^4 cell/mL in f/2 media, after which they were incubated with 0.1 μM SYTOX Green for 15 min in the dark. After addition of the chitosans at the indicated concentrations, an increase in fluorescence in response to binding of the cationic dye to intracellular DNA was monitored over time. The excitation and emission wavelengths were 485 nm and 520 nm, respectively. All fluorescence values were plotted by base fluorescence that was obtained in algal cells without chitosan [18].

Calcein leakage in artificial large unilamellar vesicles (LUVs)

Before calceine leakage assay, we extracted lipids from algal cells. The extraction of total lipids from algal cells was carried out by the solvent method [28]. Briefly, CH_2Cl_2/CH_3OH (1:1, v/v) solution was added to dried *H. akashiwo* cells and the suspension was vigorously shaken at room temperature for 5 min. After centrifugation, the supernatants were discarded and the pellets were washed until there was no green color in the supernatants. Finally, the extraction was performed using EtOAc and the extracted organic material was dried under a N_2 stream.

The permeabilization of LMWSCs against artificial vesicles was measured by calcein leakage assay. Briefly, calcein-entrapped large unilamellar vesicles (LUVs) were prepared as previously described [29]. Entrapped LUVs suspended in 5 μg/mL were incubated at the indicated LMWSC/lipid molar ratio, after which the fluorescence of the released calcein was assessed at an excitation wavelength of 480 nm and an emission wavelength of 520 nm using a spectrofluorometer (SpectraMax M5 Microplate Reader). Complete (100%) release was achieved via the addition of Triton X-100 to a final concentration of 0.03% (w/v). The apparent percentage of calcein release was calculated as follows [30]:

$$\text{Release}\ (\%) = \frac{F_p - F_0}{F_t - F_0} \times 100 \quad (2)$$

where F_p is the observed fluorescence in the presence of LMWSC, F_0 is the fluorescence of spontaneous leakage (only buffer), and F_t is the observed fluorescence after adding Triton X-100.

Confocal laser scanning microscopy (CLSM)

The cellular distribution of chitosan was examined using FITC-conjugated chitosan and CLSM. Briefly, algal cells were incubated with FITC-LMWSC-3 at IC_{90} for 5 min (*H. akashiwo* and *C. marina*) or 30 min (*P. minimum*), after which the cells were washed three times with f/2 medium and fixed with 1% (v/v) glutaraldehyde. Localization of FITC-labeled LMWSC-3 was then observed using an inverted LSM510 laser-scanning microscope (Carl Zeiss, Gőttingen,