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Preliminary analysis of allelochemicals produced by the diatom *Phaeodactylum tricornutum*

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HIGHLIGHTS

- Substances from the filtrate of *Phaeodactylum tricornutum* were separated by high-performance liquid chromatography (HPLC).
- Active compounds from *P. tricornutum* were screened using activity-guided fraction methods.
- Anti-algal compounds from *P. tricornutum* were isolated and analyzed mainly by HPLC–MS.
- The isolated allelochemical was preliminary identified as TYR-PRO-PHE-PRO-GLY-NH_2, a type of glycinamides.

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ABSTRACT

Marine diatom *Phaeodactylum tricornutum* is known to exude allelochemicals with negative effects on *Heterosigma akashiwo* according to our previous study, while the information about the allelochemical compounds remains unknown. The present study dealt with isolation and analysis of the active substances released by *P. tricornutum* into the culture medium. Filtrate of *P. tricornutum* was extracted using ethyl acetate and chloroform respectively. The anti-algal fractions were isolated using high performance liquid chromatography (HPLC) and screened using activity-guided fraction methods. Results demonstrated that fraction II and VI showed significant allelopathic effect on *H. akashiwo* growth. Then the anti-algal activity fractions were analyzed preliminary using gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography-electrospray time-of-flight mass spectrometry (HPLC-ESI-TOF-MS). An active compound was derived from fraction VI with the molecular weight of 578 and possible molecular formula of $C_{30}H_{38}N_6O_6$, which was speculated to be TYR-PRO-PHE-PRO-GLY-NH_2. a kind of glycinamides.

1. Introduction

Harmful algal blooms (HABs) have received increasing attention because they can lead to notorious consequences on human health, economies and aquatic ecosystems in both freshwater areas and marine coastal waters worldwide (Anderson, 1997; Honjo, 1994; Horner et al., 1997; Mackenzie, 1991). Allelopathy, the release of secondary metabolites (allelochemicals) that influence other phytoplankton species, is common among phytoplankton. With growing evidence, specific allelopathic interactions are considered as an important factor in phytoplankton competition for resources, succession and bloom formation (Hakanen et al., 2014; Keating, 1977, 1978; Rice, 1984; Vardi et al., 2002; Roy et al., 2009; Yang et al., 2014). Allelochemical interactions are considered as one strategy to facilitated bloom formation according to Smayda (1997), and the production and roles of allelochemicals among dinoflagellates, diatoms, chrysophyta and cyanobacteria have been reported in marine ecosystems (Graneli et al., 2012; Gross, 2003; Irfanullah and Moss, 2005; Legrand et al., 2003; Sukenik et al., 2002).

Some of the allelopathic compounds from algae and seaweeds have been isolated and characterized including alkaloids, cyclic peptides, terpenes and volatile organic compounds (Yang et al., 2014). For example, aminoacyl polyketide fischerellin A (FsA) and alkaloid 12-epi-hapalindole F (HapF) are identified as allelochemicals from *Fischerella* strain using HPLC and matrix-assisted laser-desorption ionization time-of-flight mass-spectrometry (MALDI-TOF-MS) (Etchegaray et al., 2004). A pair of chiral flavonolignans as novel anti-cyanobacterial allelochemicals are isolated from barley straw, and the allelopathic compounds are elucidated as salcolin A

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and B by nuclear magnetic resonance (NMR) and HPLC-MS (Xiao et al., 2014). The anti-algal compound-artemisinin is isolated by column chromatography and identified by GC-MS, ^{1}H NMR from the extracts of *Artemisia. annua* (Ni et al., 2012). Most of the compounds obtained above are from the cell-extraction of macroalgae or seaweeds. According to the definition of allelopathy (Rice, 1984), allelochemicals influence target species by releasing extracellular substances to the environment. Therefore, the extraction of active substances from algal cells is not proper for isolating allelochemicals even though which may take advantage of containing higher concentration of active compounds. In the past few years some researchers have made efforts to obtain active compounds even with great challenges to isolate, enrich and identify the exact structure of allelochemicals from filtrate of microalgae. For instance, Prince et al. speculated that the aromatic compound with the molecular between 500 and 1000 Da is produced by *Karenia brevis* which inhibited *Asterionellopsis glacialis* growth by allelopathy (Prince et al., 2010). Ma et al. suggested some allelochemicals produced by *Alexandrium tamarense* are suite of non-proteinaceous and non-polysaccharide substances between 7 kDa and 15 kDa using lytic hydrophilic interaction lipid chromatography separation and MALDI-TOF analysis (Ma et al., 2011).

Our previous experiments have demonstrated that *P. tricornutum* achieves growth advantage over *H. akashiwo* mainly by producing and releasing allelochemicals, however the knowledge on the information (molecular weight, property, structure, etc) of the anti-algal compounds still remains unknown. In the present study, the extracting and analyzing conditions of allelochemicals in the cell-free filtrate of *P. tricornutum* were optimized using advanced chromatography techniques and the bioactive effects of isolated fractions on *H. akashiwo* growth were verified. The active compounds were also further characterized using advanced mass spectrometry (GC-MS and HPLC -MS).

2. Materials and methods

2.1. Algal culture and preparation of filtrate

P. tricornutum was obtained from the Algal Center of Key Laboratory of Marine Chemistry Theory and Technology, Ocean University of China. Stock cultures were inoculated in f/2 medium (Guillard, 1975), prepared with autoclaved seawater of the Jiaozhou Bay of China (filtered through 0.45 μm Millipore membranes). 27 L culture was inoculated in transparent polyethylene container at (20 ± 1) °C with a 12 h/12 h light/dark cycle for every experiment. Illumination was provided by filament lamps at 70 μmol $m^{-2}s^{-1}$. Cultures were shaken manually at set time daily to avoid wall growth and prevent the sedimentation of algae.

P. tricornutum cultures were maintained until the late exponential phase, and then the culture solutions were centrifuged with a speed of 3000 r/min for 15 min. A small amounts of cells from pellet was observed with a hem cytometer under a microscope (Leica DM4000B, Germany) after centrifugation. The cells remained intact and unbroken, which indicated that no intracellular substances leaked in the process of centrifugation. Then the supernatant was filtered through 0.22 μm Millipore membranes. The obtained filtrate of *P. tricornutum* was extracted using ethyl acetate and chloroform immediately.

2.2. Lipid-lipid extraction (LLE) and high performance liquid chromatography (HPLC) analysis

27 L filtrate of *P. tricornutum* obtained above was extracted with ethyl acetate and chloroform for three times subsequently. The extracts from ethyl acetate and chloroform were pooled respectively and concentrated to 10 mL by rotary vacuum evaporator (Beijing Bo Kang laboratory instruments Medical Co., Ltd.) at 40 °C. The HPLC analysis was conducted using a Hitachi L-2000 series HPLC system (Shimadzu, Japan), equipped with a binary pump, an autosampler, a column compartment and a diode-array detector (DAD). The separation was performed on a 250 mm × 4.6 mm, 5 μm LaChrom C18 column (Hitachi, Japan) and column temperature was set as 25 °C. Mobile phase consisting of methanol (A) and water (B) and flow rate was 1.0 mL min^{-1}. The gradient program was used according to the following procedure: 0–60 min, linear increase A from 40% to 95%, and then maintained for 10 min. The UV spectra were recorded from 200 to 300 nm and the wavelength for detection was 250 nm by DAD detector. The injection volume was 20 μL.

2.3. The separation of extracellular substances of P. tricornutum and allelopathy bioassays

Different solvents such as ethyl acetate, chloroform, DMSO and n-hexane were conducted to examine the effects on *H. akashiwo* growth and only DMSO was found harmless to *H. akashiwo* growth. Moreover, different dosage of ethyl acetate extracts and chloroform extracts which were dissolved in DMSO were added into the culture medium of *H. akashiwo* (1 times, 5 times and 10 times of the approximate maximum concentration of unknown compounds in filtrate of *P. tricornutum*, respectively) for bioassays (Table 1).

The extract from the filtrate of *P. tricornutum* was isolated using HPLC, ten runs (99 μL injection volume) of samples from ethyl acetate extract were loaded on the HPLC system and nine fractions (F-I,9–11 min; F-II,25–31 min; F-III,36–39 min; F-IV,44–47 min; F-V,48 min; F-VI,50 min; F-VII,51–52.5 min; F-VIII,53.5 min; F-IX,61–64 min) were pooled according to different elution time. All the fractions were dried under N_2 and the residues were diluted with 1.0 mL DMSO respectively and stored at 4 °C before further use.

Allelopathic effect of each individual HPLC fraction on *H. akashiwo* was tested using the biological assay. The experiment was divided into two groups: (i) each 50 μL fraction was added into the culture medium of *H. akashiwo* (initial density 1.0 × 10^4 cells/mL) with the approximate maximum concentration of unknown compounds in filtrate of *P. tricornutum* to select the effective fractions. (ii) each 250 μL fraction was added into the culture medium of *H. akashiwo* (initial density 1.0 × 10^4 cells/mL) with the concentration increasing to about 5 times of the approximate maximum concentration of unknown compounds in filtrate of *P. tricornutum* to further explore the inhibition effect of higher dosage of allelochemicals on *H. akashiwo* growth. Autoclaved seawater collected from the Jiaozhou Bay of China was also extracted by LLE to serve as control and analyzed by HPLC. Using the solvent extraction and column separation methods, all the fractions were screened by their anti-algal abilities.

Table 1
Addition of different concentrations of extracts from *Phaeodactylum tricornutum*.

	Control	F_1[a]	F_1[b]	F_1[c]	F_2[a]	F_2[b]	F_2[c]
f/2 medium(mL)	150	150	150	150	150	150	150
Fractions (μL)	500 DMSO	50	250	500	50	250	500

F_1 and F_2 represent the extracts of ethyl acetate and chloroform, respectively.
[a] The approximate maximum concentration of active compound in the filtrate of *P. tricornutum*.
[b] About five times of the maximum concentration of active compound in the filtrate of *P. tricornutum*.
[c] About 10 times of the maximum concentration of active compound in the filtrate of *P. tricornutum*.