Microalgal growth enhancement by levoglucosan isolated from the green seaweed *Monostroma nitidum*

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Abstract Microalgal growth was enhanced by the addition of levoglucosan to the culture medium. The growth-enhancing compound levoglucosan was isolated from the green seaweed *Monostroma nitidum* using water extraction, molecular fractionation, DEAE-cellulose column chromatography, and high-performance liquid chromatography. Yield of the compound from seaweed powder was $5 \times 10^{-3}\%$ (w/w). At 10 mM concentration, levoglucosan enhanced cell growth and the specific growth rate of all feed microalgal species tested (*Chaetoceros gracilis*, *Chlorella ellipsoidea*, *Dunaliella salina*, *Isochrysis galbana*, *Nannochloris oculata*, *Navicula incerta*, *Pavlova lutheri*, *Tetraselmis suecica*) in most culture media by approximately 150%. Cellular fatty acid profiles and cell size differed marginally between cultures with and without levoglucosan.

Key words algal growth enhancer · Chlorophyta · levoglucosan · microalgal growth · *Monostroma nitidum*

Introduction

The mass culture of microalgae as feed for molluscs, crustaceans, and fish is an important component of the mariculture industry (Pulz & Gross, 2004). Microalgal feeds are especially valuable for early stages of marine species with fastidious dietary requirements that cannot be met by formulations of traditional agricultural products. Microalgal diets commonly have specific nutritional qualities and are in great demand because of their composition of protein, vitamins, pigments, and large amounts of docosahexaenoic acid [DHA; 22:6(n-3)] and eicosapentaenoic acid [EPA; 20:5(n-3)] (Mansour et al., 2005). Insufficient amounts of highly unsaturated fatty acids, especially DHA and EPA, cause high mortality and low growth rates, particularly during larval and early juvenile periods (Om et al., 2003; Park et al., 2006). Some microalgae have not been used extensively as nutritional diets because of difficulties in achieving high-density cultures, resulting in unreliable production (Wikfors & Ohno, 2001). The development of conventional photo-bioreactors would be one way to solve this problem (Pulz, 2001). Another way is to develop cell-growth-enhancing

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substances to increase cell biomass and thus the economic feasibility of small-scale production. Previous screening of seaweed extracts in our laboratory has shown that the aqueous extract from the green seaweed *Monostroma nitidum* Wittrock activates cell growth in several microalgae (Cho et al., 1999). In an attempt to identify the microalgal growth enhancer, we isolated the compound levoglucosan, which enhances cell growth in several microalgae in various culture media.

Materials and methods

Monostroma nitidum

Thalli of the green seaweed *M. nitidum* Wittrock (Monostromaceae, Ulvales) were collected in March 2004 and March 2005 from the upper intertidal zone of calm inlets at Imwhe (34°22'23" N, 126°15'16" E), Jindo Island, Korea. Seaweed thalli were dried completely for 1 week at room temperature and then ground to a powder for 5 min using a coffee grinder. The powder was stored at −20°C until use.

Isolation of the active compound

M. nitidum powder (100 g) was extracted three times with 5 L 100% methanol. The residual methanol was evaporated from the powder sample, after which 5 L distilled water was added to extract the water-soluble fraction for 1 day (once). The aqueous extract was evaporated using a rotary evaporator at 40°C (800 mg). The extract was then dissolved in 0.1 M Tris buffer (pH 8.0) and separated by molecular size fractionation using an Amicon diafilter membrane (Danvers, MA). An active fraction smaller than MW 500 was loaded onto a DEAE-cellulose column (50 g; 30 × 6 cm; Sigma, St. Louis, MO) equilibrated with the same Tris buffer. Elution was carried out with 500 mL of the equilibration buffer, followed by 500 mL 0.1 M NaCl in the same buffer. The 0.1 M NaCl fraction (121 mg) was separated by reverse-phase high-performance liquid chromatography (HPLC) using a μ Bondapak C-18 column (3.9 mm ID × 30 cm; Waters, Milford, MA). Analysis was performed on a Waters 600 gradient liquid chromatograph monitored at 220 nm. The mobile phase consisted of two solvent systems: acetonitrile with

0.1% TFA and distilled water with 0.1% TFA. Elution was performed using a linear gradient of 0 to 100% acetonitrile over 50 min at a flow rate of 1 mL min^{−1} for the first pass, and a linear gradient of 0 to 30% acetonitrile over 30 min for the second pass. The active growth-enhancer peak was eluted at 16% acetonitrile.

Analytical methods

The purified compound smaller than MW 500 was analyzed using a Jeol JNM-ECP 400 NMR spectrometer (Tokyo, Japan), operating at 400 and 100 MHz for ¹H and ¹³C, respectively, using methanol-d (CD₃OD). The GC-MS spectrum was analyzed using a Shimadzu GC-MS-QP5050A (Kyoto, Japan). The identity of the purified compound was confirmed by comparison with spectral data in Pfeffer et al. (1979) and Lafont et al. (1989).

Microalgal culture

To isolate the active compound, compare media, and analyze fatty acids, the axenic prasinophyte flagellate *Tetraselmis suecica* (Kylin) Butcher (CCAP-66; P-4) was cultured in f/2 medium (Guillard & Ryther, 1962) with an initial cell density of 1.2 × 10⁵ cells mL^{−1}. Purified compound (10 mM) was added to the medium and cultured under 70 μ mol m^{−2} s^{−1} light intensity at 18°C for 4 days. Cells were counted under a microscope using a hemocytometer. Specific growth rate (λ) was calculated as a cell growth against the cultured day: $\log N - \log N_0 = \lambda(T - T_0)/2.303$, where N = cell numbers at day T , and N_0 = cell numbers at day T_0 . Relative growth enhancement (%) was expressed as a relative rate: $(L/N) \times 100$, where L = cell numbers cultured with levoglucosan, and N = cell numbers cultured without levoglucosan. To examine the effect of levoglucosan, *T. suecica* cell growth was compared in six different media (100 mL): f/2, ESM, MF, MKM, M-ASP7, and W-ESM (Kasai et al.,

Figure 1 Structure of levoglucosan isolated from the green seaweed *Monostroma Nitidum*.

Figure 2 Effect of various concentrations of levoglucosan on cell growth (mean number of cells $\pm$ SD; $n \geq 3$) of the microalga *Tetraselmis suecica*. Levoglucosan was added to f/2 medium inoculated with 1.2×10^5 cells mL^{-1} *T. suecica*, and cultured at 18°C for 4 days

2004). All microalgal strains were obtained from the Korean Marine Microalgae Culture Center.

Fatty acid analysis

For fatty acid analysis, extraction of lipids and methylation were performed using methanolic boron trifluoride (Xu et al., 1998). Cells ($\sim 5 \times 10^6$) were collected from 500 mL culture by centrifugation at 1,000 g for 10 min. The algal pellet was immediately sonicated with a mixture of 8 mL water, 10 mL chloroform, and 20 mL methanol for 10 min. Then, 10 mL chloroform and 10 mL water were added and the mixture was sonicated for 10 min, respectively. After centrifugation, the lower chloroform layer was removed and evaporated under a vacuum. Lipids were methylated in 5 mL of a mixture of BF_3 (14% BF_3 in methanol), benzene and methanol (5:4:11). The tube was flushed with nitrogen gas and sealed tightly before heating at 90°C for 30 min. Fatty acid methyl

esters were extracted with GC-grade hexane and analyzed on a Shimadzu GC-MS QP5050A chromatograph equipped with a flame ionization detector.

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

Purification and identification of the active compound

The active compound was isolated as a white powdery compound (5 mg) from *M. nitidum* powder (100 g). The yield from the seaweed powder was $5 \times 10^{-3}\%$. The mass spectrum of the purified compound showed a molecular ion peak $[\text{M}^+]$ at m/z 162, which corresponded with the molecular formula $\text{C}_6\text{H}_{10}\text{O}_5$. The fragmentation pattern was compared to a library and found to match well with levoglucosan (Figure 1). The ^{13}C NMR spectrum showed the presence of six oxygenated carbons (δ_{C} 68.5, 67.8, 71.6, 72.2, 82.1, and 107.8). These oxygenated carbons were determined based on chemical shift. The ^1H NMR spectrum revealed the presence of five oxygenated methine protons and an additional methylene proton (δ_{H} 3.40, 3.49, 3.73, 3.97, 4.98, and 3.86). Based on these spectral data, the compound was identified as levoglucosan and confirmed to be identical to data for authentic levoglucosan.

Effect of levoglucosan on microalgal growth

Tetraselmis suecica attained the stationary phase of growth within 8 days in f/2 medium under 70 μmol

Table 1 Cell growth of *Tetraselmis suecica* cultured for 4 days in various media with and without 10 mM levoglucosan. Values of cell numbers are mean $\times 10^6 \pm$ SD ($n \geq 3$); $P < 0.1$

Medium	Number of cells		Specific growth rate per day	
	No levoglucosan	Levoglucosan	No levoglucosan	Levoglucosan
ESM	2.1 ± 0.1	3.1 ± 0.2	0.54	0.96
F/2	1.6 ± 0.2	2.3 ± 0.2	0.44	1.02
MF	1.9 ± 0.1	2.4 ± 0.1	0.98	1.66
MKM	3.0 ± 0.8	3.9 ± 0.2	0.18	0.30
W-ASP7	1.5 ± 0.1	2.0 ± 0.1	0.28	0.68
W-ESM	2.7 ± 0.1	4.0 ± 0.3	1.70	2.06

Table 2 Effect of levoglucosan on cell growth enhancement for various microalgae. Values of cell numbers are mean $\times 10^6 \pm$ SD ($n \geq 3$); $P < 0.1$. Each microalga was cultured for 4 days in f/2 medium with and without 10 mM levoglucosan

	Number of cells		Relative growth enhancement (%)
	No levoglucosan	Levoglucosan	
<i>Chaetoceros gracilis</i> B-015	1.2 $\pm$ 0.1	1.6 $\pm$ 0.1	133
<i>Chlorella ellipsoidea</i> C-021	1.6 $\pm$ 0.3	2.3 $\pm$ 0.4	144
<i>Dunaliella salina</i> C-248	1.0 $\pm$ 0.3	1.3 $\pm$ 0.1	130
<i>Isochrysis galbana</i> H-002	1.3 $\pm$ 0.2	1.9 $\pm$ 0.1	146
<i>Nannochloris oculata</i> C-031	1.6 $\pm$ 0.1	2.2 $\pm$ 0.1	138
<i>Navicula incerta</i> B-001	1.4 $\pm$ 0.2	1.8 $\pm$ 0.2	129
<i>Pavlova lutheri</i> H-003	1.9 $\pm$ 0.4	2.6 $\pm$ 0.3	137
<i>Tetraselmis suecica</i> CCAP-66	1.2 $\pm$ 0.2	1.9 $\pm$ 0.2	158

$\text{m}^{-2} \text{s}^{-1}$ light intensity at 18°C. The half-life of the exponential phase was approximately 4 days; thus, cell numbers were counted at 4 days to determine growth enhancement. The purified levoglucosan compound was tested at different concentrations in f/2 medium using *T. suecica* (Figure 2). At 10 mM, levoglucosan demonstrated strong growth enhancement of up to 169% relative to the reference culture without the extract (i.e., from 1.3×10^6 to 2.2×10^6 cells mL^{-1}), and this concentration was used in all subsequent experiments. To confirm the effect in various culture media, 10 mM levoglucosan (Acros organics, New Jersey, USA) was added to *T. suecica* in six different media (100 mL): f/2, ESM, MF, MKM, M-ASP7 and W-ESM, and cultured for 4 days. *Tetraselmis suecica* overall cell numbers were increased by the addition of levoglucosan to the culture media ($P < 0.1$; Table 1). Levoglucosan was most effective in media such as ESM, W-ESM and f/2, which are commonly used for marine microalgae. Both cell counts and specific growth rates of the cultures increased ($P < 0.1$). The addition of levoglu-

cosan in f/2 and W-ASP7 increased the specific growth rate of the microalga almost two-fold in the early growth phase between 3 and 4 days. Cell size (approximately 20 μm in diameter) was similar in cultures with and without levoglucosan. Other useful feed microalgae were also tested to determine the growth enhancement effectiveness of levoglucosan (Table 2). Levoglucosan enhanced the cell growth of all tested feed microalgae, especially *T. suecica*, *Isochrysis galbana* Parke, and *Chlorella ellipsoidea* Gerneck [K&H], with approximately 1.5-fold increases in cell density.

Fatty acid composition

Cellular fatty acid profiles were determined to ascertain whether the addition of levoglucosan caused changes in the lipid quality of the microalga as a feed organism. Polyunsaturated fatty acids (PUFA) are especially important in determining the nutritional value of diets for the survival and development of fish larvae. The proportions of PUFA, such as linoleic acid, linolenic acid, and EPA, differed marginally between cultures of *T. suecica* grown with and without levoglucosan (Table 3).

Table 3 Profile of major fatty acids (% of total fatty acids) in *Tetraselmis suecica* cultured for 4 days in f/2 medium with and without 10 mM levoglucosan

Fatty acids ^a	No levoglucosan	Levoglucosan
14:0	2.8	3.2
16:0	21.9	22.7
17:0	2.7	2.9
18:0	47.1	49.4
18:1 (n-9)	1.9	1.7
18:2 (n-6)	2.0	1.7
18:3 (n-3)	14.5	13.2
20:5 (n-3)	2.7	2.5

^a Peaks of <1% of the total area are omitted.

Discussion

Live algae are produced mainly in outdoor tanks or fermenters for limited local use, and the risk of contamination and maintenance costs are high. Until now, much attention has been focused on the development of new biofermenter designs (Pulz, 2001), storage methods after harvest (Lim & Park, 1998), and the improvement of nutritional value (Lopez Alonso et al., 1992). Little has been done to

develop growth enhancers to increase the productivity of live feed microalgae. We found that the cell count of cultured microalgae increased by approximately 50% when levoglucosan was added to the culture medium. However, little change occurred in the cellular fatty acid profiles and cellular size with the addition of levoglucosan. Previous research has determined that the gross biochemical composition and digestion efficiency were similar between cultures of *Isochrysis galbana* grown with and without *M. nitidum* crude extract (Cho et al., 1999). Among the 27 species of seaweed tested, only two—*M. nitidum* and *Grateloupia turuturu*—enhanced microalgal growth.

The seaweed *M. nitidum* is distributed in rocky areas in the upper intertidal zone of calm inlets. It grows during winter and early spring. It begins to mature, showing yellowish-green tissues along the thallus margin in February. Thalli are largest in March, at approximately 8 cm in length, 9 cm in width, and 2 g in wet weight. Frond cells form a monolayer. The fronds are edible and can be aquacultured using the same nets used for *Porphyra* (Kida, 1990). The aqueous extract inhibits the growth of the green alga *Enteromorpha prolifera* (Cho et al., 2001), decreases the total cholesterol and low-density lipoprotein in mouse (Jung et al., 1997), and enhances the cell growth of microalgae (Cho et al., 1999).

We isolated levoglucosan as the main active growth-enhancing compound from *M. nitidum*; the compound enhances growth for diverse microalgal species and in various culture media. Levoglucosan (CAS RN 498-07-7) contains an intramolecular glucosidic bond and has synonyms of 1,6-anhydro- β -*D*-glucopyranose, 1,6-anhydro- β -*D*-glucose, and anhydro-*D*-mannosan. Although levoglucosan can be easily hydrolyzed to glucose using an acid, none of the yeast and fungal strains tested showed levoglucosan hydrolyzing activity (Kitamura et al., 1991). In contrast, the formation of glucose 6-phosphate by an induced levoglucosan kinase was observed in reactions with cell extracts of levoglucosan-assimilating yeasts and fungi. Many eukaryotic microorganisms can utilize levoglucosan as a carbon and energy source for growth (Prosen et al., 1993; Zhuang et al., 2001). Very few prokaryotic organisms were found to use levoglucosan (Nakahara et al., 1994; Khiyami et al., 2005). Even though levoglucosan metabolism in microalgae has not been verified, its possible uses

in microalgal culture are promising because most prokaryotic microorganisms are unable to metabolize levoglucosan, whereas all microalgae tested thus far have some ability to use it as a metabolite.

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