Characterization of Protein-like Fluorophores Released from Lake Phytoplankton on the Basis of Fractionation and Electrophoresis

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Three kinds of lake plankton were cultivated, and the properties of protein-like fluorophores released from the plankton were characterized using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The results were compared with those by gel chromatography with a fluorescence detector and three-dimensional excitation-emission matrix (3-DEEM). The concentrated protein-like fluorophores of algal dissolved organic matter (DOM) were successfully separated from the fulvic-like fluorophores, and analyzed using SDS-PAGE. SDS-PAGE analysis revealed that the protein-like fluorescence DOM released from *Microcystis aeruginosa* consisted of proteins with molecular weights of 17, 37, 50, 75, 150 kDa, and greater than 250 kDa. The results of SDS-PAGE were consistent with those of gel chromatography. Those substances with molecular weights greater than 250 kDa may be a polysaccharide-peptide complex, called peptidoglycan, which is a component of bacterial cell walls. The molecular weights of protein-like fluorescence DOM from *Staurastrum dorsidentiferum* were determined to be 37 and 50 kDa. For *Cryptomonas ovata*, its DOM was found to be composed of substances with molecular weights of between 10 and 150 kDa. The results by high-performance size exclusion chromatography with chemiluminescent nitrogen detection (HPSEC/CLND) analysis suggest that the protein-like fluorophores from the plankton might be composed of substances containing organic nitrogen.

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Introduction

In Lake Biwa, the largest lake in Japan, it has been observed that the chemical oxygen demand (COD) has risen year after year since 1985, although the values of biochemical oxygen demand (BOD) and chlorophyll-*a* have been almost constant. However, the values of COD in the rivers flowing into Lake Biwa have not increased.¹ These results suggest that refractory organic matter has gradually accumulated in Lake Biwa. Seasonal and vertical variations in the concentrations of dissolved organic carbon (DOC) and organic substances, such as dissolved carbohydrates, have been examined to clarify the causes.²⁻⁴ In several lakes, chemical fractionation methods have been applied to analyze the temporal changes of DOC.⁵⁻⁹ The natural carbon stable isotope ratio of DOC has been used to identify DOC sources in aquatic systems.^{10,11} This approach was used to examine relative contributions of autochthonous and allochthonous sources to dissolved organic matter (DOM) in Lake Biwa.^{12,13}

In our previous study, DOM in Lake Biwa and rivers was investigated so as to elucidate its origins and behavior. The main origin of hydrophobic acids in Lake Biwa may be humic substances from soils around rivers that flow into Lake Biwa, while hydrophilic DOM may be due to inner production by phytoplankton.¹⁴⁻¹⁷ However, few studies about the contribution of inner production by phytoplankton to DOM have been carried out;¹⁸ therefore, three kinds of lake phytoplankton, *Microcystis aeruginosa*, *Staurastrum dorsidentiferum*, and *Cryptomonas ovata* were cultivated, and the contribution of DOM released from the phytoplankton was examined to clarify the origin of the organic pollution of Lake Biwa with regard to cultivation and biodegradation.¹⁹⁻²¹ The characteristics of the algal-derived DOM were investigated using gel chromatography with a fluorescence detector and a three-dimensional excitation-emission matrix (3-DEEM) technique. As a result, the algal DOM released from the plankton during cultivation makes an important contribution to the accumulation of the refractory organic matter in Lake Biwa. Furthermore, the fulvic-like fluorescence DOM from three kinds of plankton shows fluorescence similar in spectral distribution to that from the soil FA, but exhibits mainly hydrophilic characteristics. The protein-like fluorescence DOM released from *Microcystis*

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aeruginosa may be mainly hydrophobic DOM with a considerably large molecular weight.^{19,20}

In the present study, the properties of protein-like fluorophores released from three kinds of lake plankton during cultivation were characterized on the basis of the molecular weight using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of algal-derived DOM prepared using an ultrafiltration membrane. Furthermore, the validity of the obtained results was confirmed by comparing the results by gel chromatography with a fluorescence detector and 3-DEEM. However, since it is uncertain whether the detected bands or peaks contain organic nitrogen (N), protein-like fluorophores released from *Microcystis aeruginosa* were analyzed by using high-performance size exclusion chromatography with chemiluminescent nitrogen detection (HPSEC/CLND).²²⁻²⁴

Experimental

Reagents and apparatus

Three kinds of phytoplankton, *Microcystis aeruginosa* (NIES-109, Lake Yogo, Shiga), *Staurastrum dorsidentiferum* (NIES-665, Lake Biwa, Shiga), and *Cryptomonas ovata* (NIES-275, Tsuchiura, Ibaraki), which were supplied by the National Institute for Environmental Studies, were used.¹⁹⁻²¹

Humic and fulvic acids obtained from A-horizons of brown forest soil (Dystric Cambisol, Dando, Aichi, Japan) were used. Dando humic and fulvic acids prepared according to the methods developed by the International Humic Substances Society (IHSS) were supplied by the Japan Humic Substances Society, and used as a standard without further purification. All other chemicals were of the best commercial grade available. Pure water was prepared using a Millipore Milli-Q water purification system.

An Olympus IX71N-22PH-D microscope was used to count the number of algae cells. The apparatus used for HPLC was a Shimadzu LC-10AD chromatography pump equipped with a Shimadzu RF-10A_{XL} fluorescence detector. The excitation and emission wavelengths were 340 and 435 nm, respectively. A Shimadzu C-R7A Chromatopac was used for data analysis. The test samples were applied to a gel filtration column, Superose12 HR10/30 (300 × 10 mm i.d.; Pharmacia Biotech), using a sample injector (Rheodyne 7125) with a 100-μl loop. A 0.01 M sodium hydroxide solution was used as an eluent at a flow rate of 0.4 ml min⁻¹. A TOC meter (Shimadzu TOC-V CSH) was used for the determination of DOC. A Shimadzu UV-160A spectrophotometer was used for measuring the UV-absorbance (260 nm) of water samples. A Kubota KN-70 centrifuge and a Hitachi Koki Himac CR20GIII refrigerated centrifuge were used for the fractionation and concentration of algal DOM, respectively. A Horiba F-51 pH meter and a TOA CM-60S EC meter were used for measuring the pH and electric conductivity, respectively.

Cultivation of phytoplankton

Three kinds of phytoplankton were cultivated in an improved VT medium. *Microcystis aeruginosa* and *Staurastrum dorsidentiferum* were grown in one liter (1 L) triangular flasks at 20°C and 2000 lux under a 12 h:12 h light/dark cycle. *Cryptomonas ovata* was grown in a 1-L triangular flask at 15°C and 2000 lux under the same light/dark cycle.

The changes in time of BOD, COD, DOC, and E260 (absorbance at 260 nm) were measured in the cultivation. Dissolved organic substances were analyzed using gel chromatography with a fluorescence detector developed for the

simultaneous determination of the concentration and molecular weight of humic substances.¹⁵

The fluorescence properties of algal DOM were measured with a three-dimensional excitation-emission matrix (3-DEEM) using a fluorescence spectrophotometer, a Shimadzu RF-5300PC enabling high-resolution fluorescence measurement by running with band-pass-width slits of 5 nm for both excitation and emission. The excitation and emission wavelengths were scanned from 220 to 500 nm and from 250 to 600 nm with a wavelength scan speed of 1500 nm/min. Fluorescence readings were normalized by the fluorescence intensity (Ex = 345 nm/Em = 450 nm) of 10 μg/l quinine sulfate (0.05 M H₂SO₄ solution) 10 QSU. The values were treated as the relative fluorescence intensity (RFI).

Concentration of algal DOM using an ultrafiltration membrane and electrophoresis

On days 30 and 42 for *Microcystis aeruginosa*, 77 and 80 for *Staurastrum dorsidentiferum*, and 47 and 67 for *Cryptomonas ovata*, each 350 ml (or 200 ml) of harvested growth media including algal cells was centrifuged at 1.3 kG and 4°C for 5 min. Each supernatant was concentrated to 3 ml on a Millipore ultrafiltration membrane (molecular weight cut-off [MWCO] = 10 kDa) at a pressure of 0.5 MPa with stirring. The concentrated sample solution (3 ml) was loaded on an Econopack 10 DG column ([MWCO] = 6 kDa, Bio-Rad) to exchange the buffer solution; 3 ml of the eluate was dumped. Subsequently, the algal DOM of interest was eluted with 4 ml of a Tris-HCl buffer. A 4-ml volume of the eluate was further concentrated by using an Amicon Ultra ultrafiltration tube ([MWCO] = 1 kDa, Millipore) with centrifugation at 1.3 kG and 4°C. Finally, each 350 ml of the harvested growth media containing algal DOM was concentrated to about 1 ml, and the concentrated protein-like fluorescence DOM was fully separated from other low-molecular weight substances.

The concentrated protein-like fluorophores of algal DOM were subjected to SDS-PAGE to analyze the protein-like components. A mixture of a 16-μl concentrated sample solution and 4 μl of a loading buffer, consisting of 62.5 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.01% bromophenol blue, was heated at 80°C for 5 min. Fifteen microliters of each sample solution and 7 μl of a molecular weight marker (Bio-Rad, Precision Plus Protein standards) as standards (10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa) were loaded onto each lane of a 1-mm gel in 15% acrylamide gel (Atto PAGEL) and subjected to 300 V (20 mA) for 75 min. Subsequently, the gel was stained using the Coomassie Brilliant Blue (CBB).

Fractionation of algal DOM and 3-DEEM

Fractionation of algal DOM was carried out using Amicon Ultra ultrafiltration tubes of three different MWCOs: 30, 10, and 3 kDa. The 10-ml sample solution containing algal DOM was centrifuged on each MWCO membrane at 1.5 kG for 15 min (30 and 10 kDa) or 35 min (3 kDa). The fractionation of algal DOM (1 kDa) was carried out on a Millipore ultrafiltration filter (molecular weight cut-off [MWCO] = 1 kDa) at the pressure of 0.5 MPa with stirring, followed by the fluorescence characterization by 3-DEEM.

High-performance size exclusion chromatography and chemiluminescent nitrogen detection (HPSEC/CLND) analysis

On day 51 for *Microcystis aeruginosa*, a 100-ml of harvested growth media including algal cells was filtered through a membrane filter (0.45 μm, Millipore). The filtrate was

Table 1 Relationship between molecular weight and fluorescence intensities of the fluorophores released from three kinds of phytoplankton

Molecular weight/ kDa	Fluorescence intensity/QSU							
	Peak A			Peak B			Peak C	
	M	S	C	M	S	C	M	S
>30	3.5 (7.7)	10.6 (12.2)	9.4 (13.2)	10.1 (14.9)	18.6 (21.9)	15.6 (16.4)	165.9 (88.6)	196.9 (88.2)
10–30	1.7 (3.8)	1.7 (2.0)	0.9 (1.3)	1.8 (2.7)	2.1 (2.5)	13.5 (14.2)	2.7 (1.4)	0.3 (0.1)
3–10	4.8 (10.6)	4.8 (5.5)	7.5 (10.6)	8.6 (12.7)	1.5 (1.8)	17.5 (18.4)	1.0 (0.5)	0.8 (0.4)
1–3	19 (41.9)	24 (27.6)	53.2 ^a (74.9)	25.8 (38.0)	19.9 (23.5)	48.5 ^a (51.0)	10 (5.3)	6.1 (2.7)
<1	16.3 (36.0)	45.8 (52.7)		21.6 (31.8)	42.7 (50.4)		7.6 (4.1)	19.2 (8.6)

The values in parentheses are the ratios (%).

M, *Microcystis aeruginosa*; S, *Staurastrum dorsidentiferum*; C, *Cryptomonas ovata*.

a. <3 kDa.

concentrated to 3 ml by freeze drying. The concentrated sample solution (3 ml) was loaded on an Econopack 10 DG column ([MWCO] = 6 kDa, Bio-Rad), and then exchanged the buffer solution with 4 ml of a Tris-HCl buffer. A 4-ml volume of the eluate was further concentrated by using an Amicon Ultra ultrafiltration tube ([MWCO] = 3 kDa, Millipore) with centrifugation at 1.3 kG and 4°C. Finally, 100 ml of the harvested growth media containing algal DOM was concentrated to about 1 ml. The concentrated protein-like fluorophores of algal DOM was separated by HPSEC using a YMC-Pack Diol 200 column (a silica based column, 8 mm × 300 mm) and a HPLC system (LC-2000, JASCO) equipped with a ultraviolet (UV)-visible detector (UV-2070, JASCO) set at 280 nm, and a chemiluminescent nitrogen detector (ANTEK 8060, PAC, USA). A 50 mM sodium acetate/acetic acid buffer (pH 7) was used as the eluent. The flow rate was 1 ml min⁻¹, and at the outlet of the column, 250 µl min⁻¹ of the eluent flow was split into the CLND detector. The CLND detector conditions were as follows: argon, 50 ml min⁻¹; oxygen, 250 ml min⁻¹; ozone, 25 ml min⁻¹; oven temperature, 1050°C. The sample volume injected was 100 µl.^{23,24}

Results and Discussion

Fractionation of algal DOM

In a previous study,^{19,20} *Microcystis aeruginosa*, *Staurastrum dorsidentiferum*, and *Cryptomonas ovata* were cultivated, and the contribution of dissolved organic matter (DOM) released from the phytoplankton was evaluated by gel chromatography with a fluorescence detector (Ex = 340 nm, Em = 435 nm). As a result, the algal DOM released from plankton during cultivation mainly gives rise to a significant peak (M_w : <3 kDa). Since this peak is in good agreement with one of three chromatographic peaks clearly detected in the surface water of Lake Biwa, it can be concluded that the algal DOM released from the plankton during cultivation makes an important contribution to the refractory organic matter in Lake Biwa. From the spectral base characterization, the cultivation of three kinds of phytoplankton was found to yield two fulvic-like fluorescence peaks (A and B)

Fig. 1 3-DEEM contour plots of algal DOM from *Microcystis aeruginosa* before (a) and after (b) the gel filtration using an Econopack 10-DG column ([MWCO] = 6 kDa). A, B, fulvic-like fluorescence peaks; C, protein-like fluorescence peak.

and a protein-like fluorescence peak (C). The fulvic-like fluorescence DOM (peak A) from *Microcystis aeruginosa* may be due to the relatively small molecular weight, as compared with those of the soil FA, while protein-like fluorescence DOM (peak C) is anticipated to be arising from a considerably large substance.¹⁹

To clarify this, first, algal DOM from three kinds of plankton was fractionated by the ultrafiltration and characterized in detail in the present study (Table 1). Table 1 summarizes the relationship between the molecular weight of the components and their fluorescence properties. The molecular weight of over 70% of fulvic-like fluorescence DOM (peaks A and B) from the plankton was estimated to be less than 3 kDa. These results were consistent with those obtained by gel chromatography. The molecular weight of protein-like fluorescence DOM (peak C) for *Microcystis aeruginosa* and *Staurastrum dorsidentiferum* was estimated to be over 30 kDa. Gel chromatograms of algal DOM from *Microcystis aeruginosa* at Ex/Em wavelength of peak C showed a large peak at retention time (RT) = 17–18 min and several small peaks at 32, 35, and 47 min. Based on the calibration curve for molecular-weight standards, the peaks

Fig. 2 SDS-PAGE analysis of the concentrated algal DOM released from *Microcystis aeruginosa* before (a) and after (b1, b2) the gel filtration using a 10-DG column. STD, marker; cultivation, 30 days.

detected at 17–18, 32, and 35 min corresponded to the molecular weights of about 200, 3–5, and <3 kDa, respectively.

SDS-PAGE of concentrated protein-like fluorescence DOM from *Microcystis aeruginosa*

No bands were detected in the algal DOM without any concentration-treatment with SDS-PAGE. Thus, the untreated algal DOM was considered to contain a considerably small amount of protein-like fluorescence DOM. Therefore, we used ultrafiltration for concentration, and exchanged the VT media with a Tris-buffer to remove interfering substances by using 10-DG column prior to the SDS-PAGE analysis. 3-DEEM contour plots of algal DOM from *Microcystis aeruginosa* before and after gel filtration using a 10-DG column are shown in Fig. 1. Two fulvic-like fluorescence peaks (A and B) and a protein-like fluorescence peak (C) were detected before gel filtration, but it should be noted that only a protein-like fluorescence peak (C) was detected after gel filtration. These results indicate that only protein-like fluorescence DOM can be isolated from fulvic-like fluorescence DOM with this step.

The SDS-PAGE patterns of the concentrated algal DOM from *Microcystis aeruginosa* (cultivation 30 days) before and after the gel filtration using a 10-DG column are shown in Fig. 2. The observation that the lane of the sample before the gel filtration gave rise to an unclear band pattern might be due to many contaminants. Conversely, clear bands corresponding to the positions at approximately 17, 37, 50, 75, and 150 kDa were observed in the lanes of the buffer exchanged sample after the gel filtration, and the bands around 37 and 150 kDa were fairly clear. The protein-like fluorescence DOM from *Microcystis aeruginosa* may comprise a mixture of substances with diverse MWs. Since a clear band was observed at the top of the sample lane, the protein-like fluorescence DOM is considered to consist of substances with a molecular weight greater than 250 kDa. This is consistent with the results of gel chromatography that

Table 2 Molecular weights of protein-like fluorescence substances released from three kinds of phytoplankton by SDS-PAGE

Phytoplankton	Cultivation/day	Molecular weight of protein/kDa
<i>Microcystis aeruginosa</i>	30	17, 37, 50, 75, 150, >250
	42	15–20, 50, 150, >250
<i>Staurastrum dorsidentiferum</i>	77	37, 50
	80	37, 50
<i>Cryptomonas ovata</i>	47	50–150
	67	10, 15, 37, 75, 150

the protein-like fluorescence DOM from *Microcystis aeruginosa* had a large molecular weight of greater than 200 kDa.¹⁹ The algal DOM from *Microcystis aeruginosa* on day 42 had molecular weight of between 15 and 20, 50, 150 kDa and more than 250 kDa (Table 2). The substances with large molecular weights might be peptidoglycans, which are components of bacterial cell walls.²⁵

Since mass propagation of algae in drinking-water source affects the coagulation process in water-treatment systems, it is highly desirable to establish effective countermeasures against such coagulation inhibition. In this context, it is crucial to identify any causative substances released from algae.^{26,27} *Microcystis aeruginosa* was cultivated, and recovered organic matters from the culture were analyzed using gel chromatography and SDS-PAGE.^{28,29} The results of gel chromatography showed that algal organic matters (AOMs) have various molecular weights of between 10 and 20 kDa, around 40 kDa, and greater than 600 kDa. SDS-PAGE analysis showed that AOMs included components that are able to form complexes with aluminum; such components have molecular weights between 43 and 67 kDa.^{28,29} These results are in good agreement with ours as stated above.

SDS-PAGE of concentrated protein-like fluorescence DOM from *Staurastrum dorsidentiferum* and *Cryptomonas ovata*

The SDS-PAGE electrophoretograms of the concentrated algal DOM from *Microcystis aeruginosa* (M), *Staurastrum dorsidentiferum* (S), and *Cryptomonas ovata* (C) are shown in Fig. 3. The molecular weights of protein-like fluorescence substances released from the three planktons by SDS-PAGE analysis are listed in Table 2. Two clear bands corresponding to the positions at 37 and 50 kDa were observed in the lane of the concentrated algal DOM from *Staurastrum dorsidentiferum*, and the band at 50 kDa was much clear. A smear electrophoretic band pattern split into several bands at approximately 10, 15, 37, 75, and 150 kDa was observed in the lane of the concentrated algal DOM from *Cryptomonas ovata* on day 67. Several unclear bands between 50 and 150 kDa were observed in the lane of the concentrated algal DOM from *Cryptomonas ovata* on day 47. As the band at greater than 250 kDa was not observed at the top of each lane for *Staurastrum dorsidentiferum* and *Cryptomonas ovata*, substances with a large molecular weight of greater than 250 kDa may not be present in both algal DOM, which significantly differs from the case of *Microcystis aeruginosa*. The bands of *Cryptomonas ovata* were not clear relative to those of *Microcystis aeruginosa* and *Staurastrum dorsidentiferum*. This is possibly not only because the protein-like fluorescence substances released from *Cryptomonas ovata* might have been decomposed, but also because those released from *Microcystis aeruginosa* and *Staurastrum dorsidentiferum* could be relatively refractory.³⁰ Furthermore, the effects of cultivation days and

Fig. 3 SDS-PAGE analysis of the concentrated algal DOM from *Microcystis aeruginosa* (M), *Staurostrum dorsidentiferum* (S) and *Cryptomonas ovata* (C) during cultivation for 30, 77 and 67 days, respectively.

preconcentration on the properties of protein-like fluorescence DOM should be examined because their molecular weights, and fluorescence may change during cultivation or the process of preconcentration.

HPSEC/CLND analysis of concentrated protein-like fluorescence DOM from *Microcystis aeruginosa*

Furthermore, to make clear that the protein-like fluorophores of algal DOM contain organic N, HPSEC/CLND was applied to analyze the concentrated protein-like fluorescence DOM released from *Microcystis aeruginosa*. By HPSEC/CLND analysis, each N-containing compound eluted from an HPLC column was introduced into a furnace at 1050°C and oxidized to nitric oxide (NO), and then determined using a chemiluminescence detector with ozone.^{22,23} Figure 4(a) shows chromatogram of protein standards by HPSEC/CLND analysis. Thyroglobulin (660 kDa), ovalbumin (45 kDa), myoglobin (17 kDa) and vitamin B-12 (1.4 kDa) were detected at 6.2, 8.5, 10.9 and 11.9 min, respectively. The chromatograms of the concentrated protein-like fluorescence DOM from *Microcystis aeruginosa* recorded by CLND and by UV absorbance at 280 nm are shown Figs. 4(b) and 4(c), respectively. A significant peak at 6.1 min and several small peaks between 7 and 11 min were observed in both chromatograms, showing that N-containing DOM has molecular weights ranging from 10 to 660 kDa. A large peak between 12 and 20 min observed in the N chromatogram from the HPSEC/CLND analysis is likely to be attributed to Tris-HCl buffer in which the concentrated algal DOM was dissolved. From these results, the protein-like fluorescence DOM from plankton might be composed of polypeptide-like substances containing organic N. An analysis of the protein-like fluorescence DOM, including the determination of the N-amino acid sequences, will be conducted in the further study.

Fig. 4 High-performance size-exclusion chromatograms of protein standards (a) and the concentrated algal DOM from *Microcystis aeruginosa* on day 51 (b) (c) recorded by different detection methods. (a), (b) Chemiluminescent nitrogen detector (CLND), (c) UV detector at 280 nm. Injection volume: (a) 10 µl; 1, thyroglobulin (660 kDa, 1.9 µg); 2, ovalbumin (45 kDa, 3.6 µg); 3, myoglobin (17 kDa, 2.3 µg); 4, vitamin B-12 (1.4 kDa, 0.32 µg), (b) (c) 100 µl.

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