

Isolation and purification of the major photosynthetic antenna, fucoxanthin-Chl *a/c* protein, from cultured discoid germlings of the brown Alga, *Cladosiphon okamuranus* TOKIDA (Okinawa Mozuku)

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Abstract A chlorophyll *c* binding membrane intrinsic light-harvesting complex, the fucoxanthin-chlorophyll *a/c* protein (FCP), was isolated from cultured discoid germlings of an edible Japanese brown alga, *Cladosiphon* (*C.*) *okamuranus* TOKIDA (Okinawa Mozuku in Japanese). The discoid germling is an ideal source of brown algal photosynthetic pigment-protein complexes in terms of its size and easiness of cultivation on a large scale. Ion-exchange chromatography was crucial for the purification of FCP from solubilized thylakoid proteins. The molecular weight of the purified FCP assembly was estimated to be ~56 kDa using blue native-PAGE. Further subunit analyses using 2D-PAGE revealed that the FCP assembled as a

trimer consisting of two distinguishable subunits having molecular weights of 18.2 (H) and 17.5 (L) kDa. Fluorescence and fluorescence-excitation spectra confirmed that the purified FCP assembly was functionally intact.

Keywords Photosynthetic light-harvesting complex · Membrane protein · *Cladosiphon okamuranus* TOKIDA · Brown algae · Discoid germling

Abbreviations

BN-PAGE	Blue native-PAGE
<i>C.</i>	<i>Cladosiphon</i>
CBB	Coomassie brilliant blue
Chl	Chlorophyll
DM	<i>n</i> -dodecyl- β -D-maltopyranoside
FCP	The fucoxanthin-chlorophyll <i>a/c</i> protein

Introduction

Oceanic photosynthetic organisms, such as diatoms, some prokaryotes and brown algae, contribute a large fraction of the primary production on Earth (Wright and Jeffrey 2005; Uitz et al. 2010). They possess rather unique membrane-intrinsic light-harvesting complexes, the chlorophyll (Chl) *a/c* type antennas. These light-harvesting complexes are genetically similar to the Chl *a/b* type antenna complexes found in green plants. The Chl *a/c* complexes have been relatively poorly studied compared with their higher plant counterparts (Macpherson and Hiller 2003). The most abundant Chl *a/c* type antenna, the fucoxanthin-Chl *a/c* protein (FCP), purified from a diatom *Cyclotella meneghiniana* has been relatively well studied (Beer et al. 2006; Büchel 2003; Veith et al. 2009). Büchel et al. introduced ion-exchange chromatography in addition to a well-used

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sucrose density gradient centrifugation step to separate the FCPs into two different forms: one is a trimer that consists of 18 kDa subunit and the other is a higher oligomer that has 19 kDa subunits (Büchel 2003). In contrast, brown algal FCP assemblies are much more sensitive to the detergents than those from diatoms, and so the details of their functional units have not been established (Katoh and Ehara 1990; Katoh et al. 1989). Purification of brown algal FCPs, using isoelectric focusing (IEF), revealed the presence of another minor light-harvesting complex that has a high content of xanthophyll cycle carotenoids (De Martino et al. 2000, 1997). Clearly there is a need to study the brown algal light-harvesting system in much more detail.

A major problem in the purification of brown algal photosynthetic pigment-protein complexes is the development of mild extraction procedures that can isolate thylakoid membranes from very tough algal cells. In this study we have taken advantage of using *Cladosiphon* (*C.*) *okamuranus* TOKIDA, which is an edible brown alga that comes from Okinawa, Japan. *C. okamuranus* can produce discoid germilings. Discoid germilings are micro-scale precursors of algal bodies (see Fig. 1 for a photograph taken by an optical microscope). They are rich in photosynthetic membranes and lack the tough outer body of lipids and polysaccharides. This then allows milder methods to be used in isolating thylakoid membranes. Methods for mass production of the discoid germilings have been described (Iinuma et al. 2009).

In this study we describe characterization of FCPs isolated from discoid germilings of *C. okamuranus*.



Fig. 1 A microscopic image of a discoid germiling from *C. okamuranus* TOKIDA. The scale bar indicates 40 μm

Materials and methods

Mass production of discoid germilings from *C. okamuranus* (Iinuma et al. 2009)

The spores of plurilocular sporangia of wild-type *C. okamuranus* were collected from the shore at Katsuren, Uruma, Okinawa, in June 2007. Initial and pre-cultures of discoid germilings were produced by the hanging drop method. The discoid germilings were grown for 1 week in TSP₂₇ medium (South Product Ltd., Uruma, Okinawa, Japan) at 25°C under a 12 h light – 12 h dark cycle. The cells were illuminated by a white light from a fluorescent light (Neolumi Super FL40SD, Mitsubishi Electric Osram Ltd., Yokohama, Japan) at 80 μmol of photosynthetic photon flux (400–700 nm) $\text{m}^{-2} \text{s}^{-1}$ (ap. 5000 lx). Ten kg of the discoid germilings could be harvested within 2 months starting from one gram the original culture. After harvesting the discoid germilings were frozen and stored at -30°C before use.

Solubilization of thylakoid proteins

Thylakoid membranes were isolated from discoid germilings using essentially the same method that has been previously described for diatoms (Beer et al. 2006; Büchel 2003). In brief, the discoid germilings were resuspended in buffer A (10 mM MES pH 6.5, 2 mM KCl, 5 mM EDTA) and passed through a French press (Ohtake, LLC, Tokyo, Japan) at 150 MPa. Cell debris were removed by a centrifugation (12,000 g for 20 min, 4°C), and membranes were pelleted by 1 h of another centrifugation at 7,500 g. The membrane fraction was then washed with buffer A by a further centrifugation step (40,000 g for 20 min, 4°C) and resuspended in a little amount of buffer B (25 mM Tris. HCl pH 7.5 with 2 mM KCl). Solubilization of the membranes was performed in buffer B at a concentration of Chl *a* of 0.25 mg ml^{-1} by the addition of 10 mM *n*-dodecyl- β -D-maltopyranoside (DM, Kishida Chemical Co. Ltd., Tokyo Japan). This solubilization was carried out for 10 min in the dark on ice. The mixture was then centrifuged at 15,000 g for 10 min at 4°C. The brown supernatant was collected and used for further purification.

Purification of the FCP

Ion-exchange chromatography

The solubilized thylakoid proteins were loaded onto the top of an ion-exchange column (DEAE Sepharose fast flow, GE Healthcare, Japan) that had been equilibrated with buffer B containing 0.03% (w/v) DM (now called buffer C). A linear gradient of NaCl in buffer C was applied,

as shown in Fig. 2a, to elute the complexes. Brown fractions with ratios of the absorbance at 530 nm: 437 nm > 0.26 were collected.

Gel-filtration chromatography

The crude FCPs fractions from the ion-exchange chromatography were injected into a gel-filtration column (Hi-Load superdex G200, GE Healthcare) that had been equilibrated with buffer C at 4°C. The column was run with buffer C at 4°C using an FPLC (Akta prime, GE Healthcare). Fractions were collected based on the criterion of the relative absorbance described above.

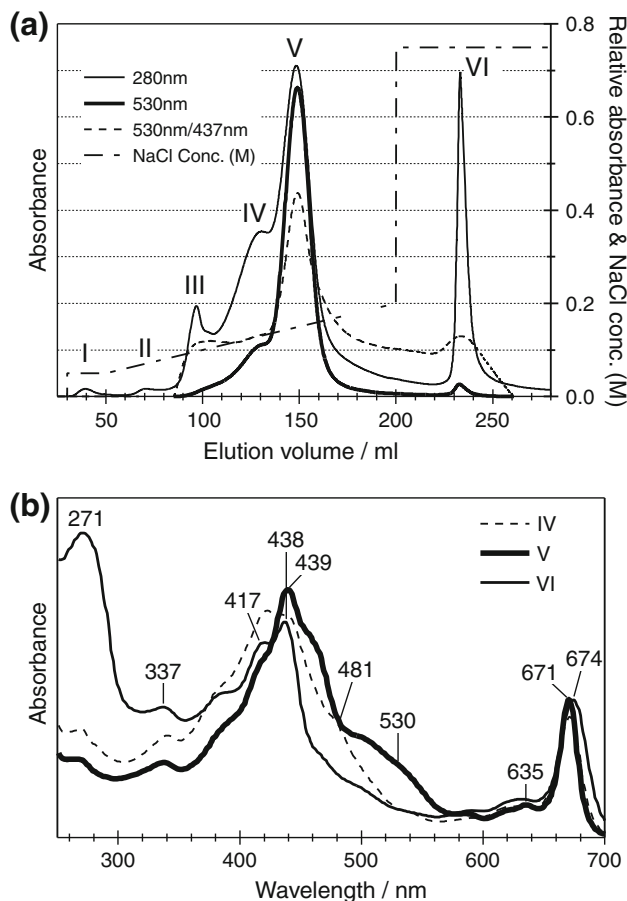


Fig. 2 **a** Elution profiles of the thylakoid proteins of the discoid germling from *C. okamuranus*, obtained using ion-exchange chromatography monitored at 280 nm (thin solid line) and 530 nm (thick solid line). The relative absorbance A_{530}/A_{437} is also plotted (broken line). Concentration of NaCl in buffer C (25 mM Tris, HCl pH 7.5, 2 mM KCl, 0.03% DM) is shown with the dash-dotted line. **b** Absorption spectra of the major protein peaks fractionated by the ion-exchange chromatography; IV undetermined pigment-protein complex with free pigments; V fucoxanthin-chlorophyll *a/lc* protein; VI photosystems

Sucrose density gradient centrifugation

The sucrose gradients were prepared by a freeze-and-thaw treatment of buffer C containing 19% (w/v) sucrose (Büchel 2003). The brown supernatant collected from the initial solubilization was layered on top of the sucrose gradient. The gradient was centrifuged at 257,000 g for 15 h at 4°C. The central part of the brown band was collected. This method was only used during the development of purification methods (see [Development of a purification method for the preparation of intact FCP assemblies from discoid germlings](#), section).

Characterization of purified pigment protein complexes

Electrophoresis

For denaturing SDS-PAGE, a ready-made gel (Tris, HCl pH 8.8, 12.5% or 15.0% (w/v) acryl amide, Bio-Rad Laboratories, K.K., Tokyo, Japan) was used. The protein solutions were suspended in Laemmli sample buffer (Laemmli 1970) (Bio-Rad Laboratories) and 5% (v/v) 2-mercaptoethanol (Bio-Rad Laboratories), and were denatured by heating at 37°C for 30 min before being loaded onto the precast gel. SDS-PAGE was performed according to Laemmli's standard method (Laemmli 1970) at 40 mA for 90 min at 4°C by using a Tris-glycine-SDS buffer (Bio-Rad Laboratories), and stained with bio-safe Coomassie brilliant blue (CBB) G-250 (Bio-Rad Laboratories).

For non-denaturing blue native (BN)-PAGE, a 5 → 12% acryl amide gradient gel was prepared according to the protocol of (Witteig et al. 2006). The gel was buffered with 25 mM of imidazole at pH 7.0. The protein solutions were diluted twice with a solubilization buffer (25% (w/v) glycerol, 0.5% (w/v) CBB G-250, 50 mM Imidazole pH 7.0), and then solubilized with 1% (w/v) DM for 5 min on ice before being loaded onto the gradient gel. BN-PAGE was performed at 4 mA for 1–3 h at 4°C. The cathode buffer consisted of 50 mM of tricine, 7.5 mM of imidazole, 0.05% (v/v) Triton X-100, 0.02% (w/v) CBB G-250, whereas anode buffer was 25 mM imidazole, pH 7.0 (Witteig et al. 2006).

Size-exclusion chromatography

Size-exclusion chromatography was carried out using an HPLC system with a TSK-gel column G4000PW_{XL}, 7.8 i.d. × 300 mm, TOSOH, Kyoto, Japan, equilibrated with buffer B containing 0.075% (w/v) DM (buffer D). The flow rate was 0.5 mL min⁻¹ and the elution fractions monitored in the wavelength range of 300–700 nm. The column was run at 10°C.

N-terminal sequencing

The two subunits, separated by SDS-PAGE (see above) were transferred to a PVDF membrane ($9 \times 5.5 \text{ cm}^2$, Immobilon-P[®], Millipore, Billerica, MA) by applying a current of 2 mA for each 1 cm^2 (100 mA in total) for 1 h as described in (Ursitti et al. 2001). The membrane was stained with Amido black 10B. N-terminal sequencing of the bands was carried out by APRO Life Science Institute, Inc., Tokushima, Japan.

Spectroscopy

Absorption, fluorescence, and fluorescence-excitation spectroscopy were carried out using V-670, Jasco, Japan and F6000, Jasco spectrometers. All spectra were recorded at 4°C in buffer C. The fluorescence emission spectra were recorded using excitation at 437 nm. The fluorescence-excitation spectrum was recorded monitoring fluorescence at 730 nm. The power of the excitation light was calibrated from 400 to 1100 nm using a combination of rhodamin 6G and a secondary-standard illuminant unit (ESC-333, Jasco) for the fluorescence-excitation measurement.

Results and discussion

FCP proteins isolated from discoid germlings from *C. okamuranus*

Büchel's group have successfully improved the quantity and quality of the FCP complexes from a diatom, *Cyclotella meneghiniana* by introducing an ion exchange chromatography instead of (Beer et al. 2006) or before (Gundermann and Büchel 2008) sucrose density centrifugation. Since this method has never been tested for the preparation of brown algal FCP, we tested the applicability of this method.

Figure 2a shows the ion-exchange chromatographic profiles of DM-solubilized thylakoid proteins from *C. okamuranus*. The first three peaks eluted at 50–100 mM NaCl (bands I–III) could be attributed to free pigments. The last peak eluted at 750 mM NaCl (band VI) was characterized by a smaller A530/A437 ratio of 0.14 compared to that of band V, indicating a small amount of bound fucoxanthin in relation to Chl *a* as described in Beer et al. (2006). The band VI exhibited a comparable absorption spectrum (shown by a thinner solid line in Fig. 2b) as described for isolated photosystem II from diatom (Nagao et al. 2007). Therefore, band VI was not considered further. Band V exhibited the largest A530/A437 ratio of 0.42, indicating the largest content of bound fucoxanthin in relation to Chl *a* (Beer et al. 2006). This band showed an

absorption spectrum comparable to that of FCP (Beer et al. 2006; Büchel 2003; De Martino et al. 2000, 1997; Veith et al. 2009) and was, therefore, assigned to FCP. Band IV had a smaller A 530/A437 ratio of 0.12 and its absorption spectrum (shown as broken line in Fig. 2b) showed a shoulder at 481 nm, indicating a smaller amount of fucoxanthin and the presence of different carotenoid (possibly violaxanthin). These spectral features resemble those found in the case of the minor brown algal antenna complexes that contain violaxanthin (Barrett and Anderson 1980; De Martino et al. 1997). However, since this complex is so unstable it was not possible to study it further.

Development of a purification method for the preparation of intact FCP assemblies from discoid germlings

Three different methods were compared for their suitability to produce stable purified FCP assemblies: (A) ion-exchange chromatography (IE) after a sucrose gradient (SG); (B) gel-filtration (GF) after IE; (C), GF after SG. The purity of the FCP assemblies after each of the three purification methods were evaluated using SDS-PAGE, blue native (BN)-PAGE and size-exclusion chromatography.

The results of these analyses together with the absorption spectra of the different fractions are shown in Fig. 3. The broad band in the SDS-PAGE at $\sim 18 \text{ kDa}$ could be assigned to monomeric FCP apo-proteins (Caron et al. 1985; De Martino et al. 2000, 1997; Durnford et al. 1996; Katoh and Ehara 1990; Katoh et al. 1989; Macpherson and Hiller 2003). The faint green band around 10 kDa was due to free pigments (designated as f.p. in Fig. 3a). There were some higher molecular weight impurities seen in the lanes (designated as an asterisk in the figure) corresponding to the fractions produced by the purification methods A and C. These impurities were much less in the case of the purification method B. A broad greenish band seen in the BN-PAGE located below the 66 kDa marker can be tentatively assigned to be the FCP assembly (designated as FCPa in the Fig. 3b), because this band appeared in all three samples. This band is rather clear with purification method B, whereas this band shows quite a long tail to higher molecular weights in both purification methods A and C. This can be explained by the presence of an impurity having molecular weight around 100 kDa (designated by asterisk) that was removed by purification method B. A deep blue band around $18\text{--}20 \text{ kDa}$ in Fig. 3b may be the stain (CBB) judged by the band shape. Therefore, this data suggests that the intact FCP complex isolated from *C. okamuranus* is a trimer of the monomeric subunits.

Size-exclusion chromatography of the fraction produced by the purification method B shows one distinct peak at an elution volume of 8.4 ml (Solid line in Fig. 3c). On the

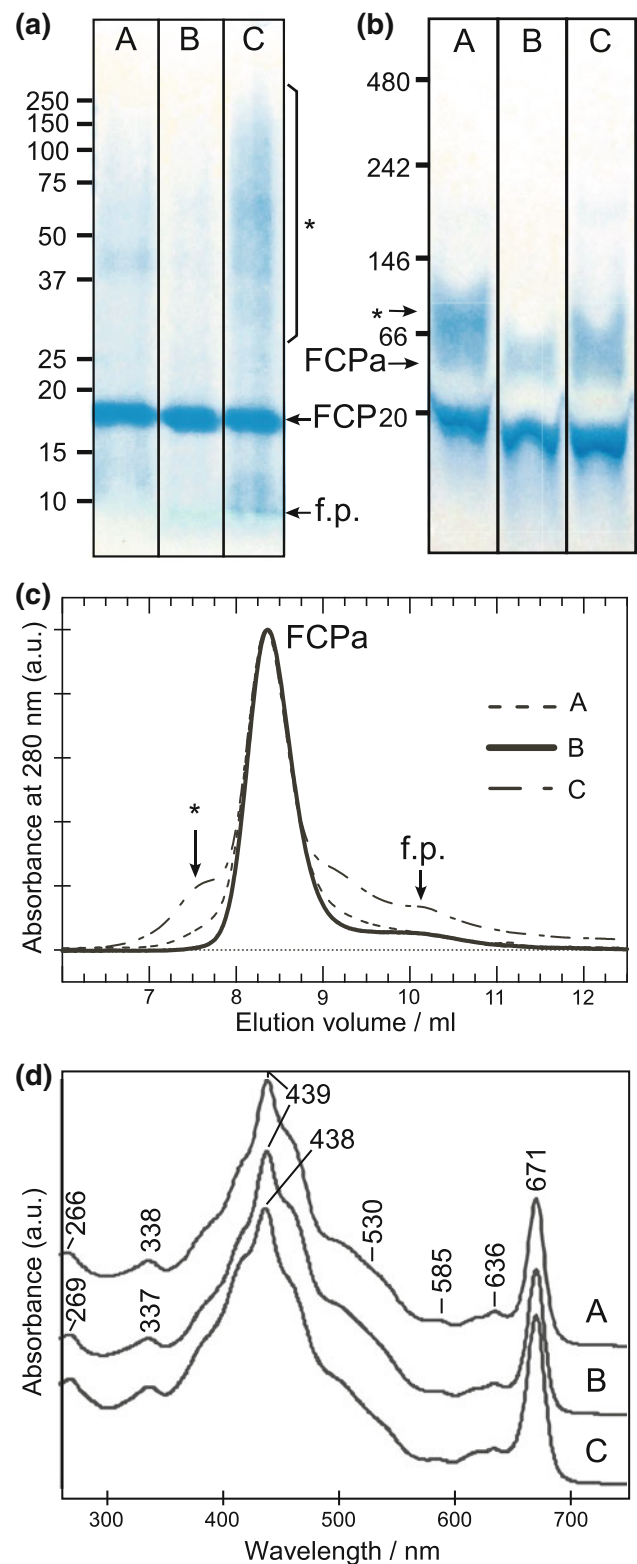
Fig. 3 **a** SDS-PAGE (12.5% stained by CBB), **b** BN-PAGE (4–12% gel stained by CBB), **c** size exclusion chromatography (TSK-gel G4000 PW_{XL}), and **d** absorption spectra of the FCP purified by different methods: **A** ion-exchange chromatography (IE) after sucrose gradient (SG); **B** gel-filtration (GF) after IE; **C** GF after SG. FCP, FCP subunits; FCPa, FCP assembly; f.p., free pigments; Asterisks in **b**, **c** indicate the location of an impurity having molecular weight of ~100 kDa

basis of its absorption spectrum (data not shown) this peak can be assigned to the FCP assembly. When the FCP assembly was solubilized with 5% (w/v) of DM before injecting to the size-exclusion column, the small plateau around an elution volume of 10 ml was much enhanced (data not shown). Therefore, the small plateau can be due to free pigments (designated as f.p. in the figure). The absorption spectrum of the small plateau supports this assignment (data not shown). The size-exclusion profiles of the fractions obtained from purification methods A and C (broken and dash-dotted lines in Fig. 3c, respectively) show two additional peaks at elution volumes around 7.5 and 9.1 ml. The elution order in size-exclusion chromatography correlates with molecular weights; the larger a protein is, the faster it elutes. The calibration of size (molecular weight) in relation to the elution volume of the size-exclusion chromatography is difficult for the membrane proteins because the proteins produce detergent micelles in the solution. However, we can ascribe the former (latter) band for larger (smaller) molecular weight than the FCP assembly. Therefore, these additional peaks at elution volumes around 7.5 and 9.1 ml can be assigned to the ~100 kDa impurity found in the BN-PAGE and the monomeric subunit of FCP (~20 kDa band), respectively. Purification method B, therefore, produces the purest FCP assembly. These three biochemical analyses revealed that the purity of the FCP assembly was higher in the order, Method B > Method A > Method C. Clearly, the ion-exchange chromatography step was crucial for the purification of the brown algal FCP. We henceforth chose purification method B for the isolation of FCP from the discoid gemilings of *C. okamuranus*.

The absorption spectra of the FCP assembly purified by these three methods are compared in Fig. 3d. The relative absorbances of A_{530}/A_{437} for the fraction using purification method C was 0.2, whereas those for A and B were both 0.3. The latter value is consistent with the value of the FCP isolated from diatoms (Beer et al. 2006), although this criteria is not a good criterion of purity.

Analysis of the apo-protein composition of the FCP assembly

The broad ~18 kDa band seen in the SDS-PAGE results described above could be occasionally seen to contain two



bands. Therefore, this band was analyzed further using a 2D system. First the FCP assembly was run on the BN-PAGE and then this band was resolved by SDS-PAGE. The results are shown in Fig. 4. When purified FCP assemblies

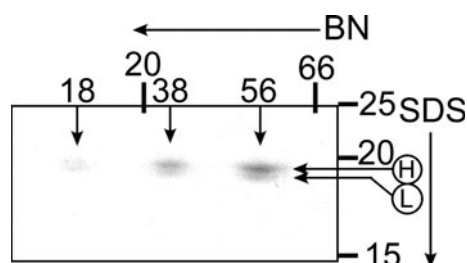


Fig. 4 Result of 2D-PAGE: BN-PAGE was followed by SDS-PAGE. Markers running with both PAGES were designated in both axes in kDa

were solubilized with 2% (w/v) DM (see experimental). BN-PAGE showed three bands with molecular weights at ~ 56 , ~ 38 , and ~ 18 kDa (see Fig. 4). The second dimension clearly shows that each of the bands from the BN-PAGE contained subunits with molecular weights of 18.5 (H) and 17.2 (L) kDa. On this basis, the bands with molecular weights of ~ 56 , ~ 38 , and ~ 18 kDa in the BN-PAGE could be assigned as trimeric FCP, dimeric FCP, and monomeric FCP, respectively. This shows that the trimeric FCP can be dissociated with higher concentrations of DM.

N-terminal sequences

The N-terminal sequencing of the H and L subunits was difficult because of blocked N-termini. Because of this only nine aminoacids from the L peptide could be assigned. This sequence shows 100% identity when compared to FCP proteins from other brown algae such as *Macrocystis pyrifera* (Apt et al. 1994, 1995), *Laminaria (L.) saccharina* (Caron et al. 1996), *L. digitata* (De Martino et al. 2000). Further search for the FCP proteins uploaded in the data bases (GenPept, RefSeq, Swiss-Prot, PIR, PRF, and PDB) revealed up to 78% identity for the FCP proteins from some diatoms, *Phaeodactylum tricornutum* (Apt et al. 1994), *Cylindrotheca fusiformis* (Poulsen and Kröger 2005), and *Odontella sinensis* (Kroth-Pancic 1995) and a raphidophyceae algae, *Heterosigma carterae* (Durnford et al. 1996). This high degree of homology clearly identifies the L subunit of *Cladosiphon okamuranus* as a *bona fide* FCP subunit.

Energy transfer within the purified FCP assembly

The purified FCP assembly was intact with regard to excitation energy transfer to Chl *a* as proven by their fluorescence excitation and emission spectra shown in Fig. 5. The fluorescence emission spectrum excited at 438 nm (broken line) showed a peak at 676 nm with a vibrational progression at 735 nm, due to the bound

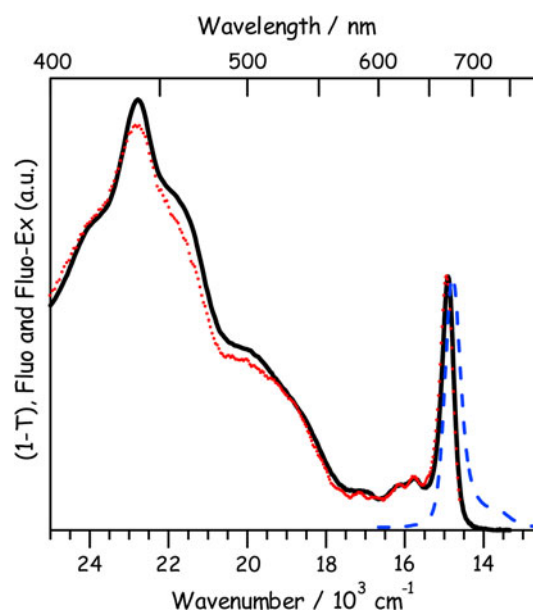


Fig. 5 Fluorescence (broken line) and fluorescence-excitation (dots) spectra of trimeric FCP from *C. okamuranus* at 4°C. Absorbance (1-T) was also shown as a solid line. All the spectra are normalized at maximum around 671 nm. Excitation (probed) wavelength for fluorescence (fluorescence-excitation) spectrum was 438 nm (730 nm)

Chl *a*. The singlet-singlet energy transfer efficiencies from antenna pigments, fucoxanthin and Chl *c*, to Chl *a* in the FCP were evaluated by comparing the magnitude of the fluorescence-excitation and absorbance (1-T) in the specific wavelengths where the antenna pigments absorb (Kosumi et al. 2010). Monitoring the fluorescence of Chl *a* at 730 nm, the fluorescence-excitation spectrum (dots) showed excellent agreement with the absorbance, indicating almost 100% energy transfer when fucoxanthin, 515–550 nm, or Chl *c*, 600–640 nm, were excited. This is in good agreement with previous studies with FCP which showed strong coupling between fucoxanthin and Chl *a* as well as between Chl *c* and Chl *a* (Macpherson and Hiller 2003). Relatively small energy transfer of $\sim 90\%$ in 430–515 nm region was observed from Fig. 5, suggesting the presence of another type of fucoxanthin (blue Fx) which is only weakly energetically coupled to Chl *a* (Gildenhoff et al. 2010; Macpherson and Hiller 2003; Premvardhan et al. 2009).

Conclusion

The FCP assembly was purified from cultured discoid germlings from *C. okamuranus* by a combination of ion-exchange chromatography and gel-filtration. The FCP exists as a trimer consisted of two subunits having 17.5 and 18.2 kDa molecular weights. N-terminal sequence

indicates that the FCP can be regarded as a typical brown algal FCP and be distinguishable from the FCP from diatoms. Fluorescence-excitation spectrum revealed extremely high (90–100%) excitation energy transfer to Chl *a*, confirming that the purified trimeric FCP was functionally intact.

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