Isolation and characterization of oxygen-evolving thylakoid membranes and Photosystem II particles from a marine diatom *Chaetoceros gracilis*

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Abstract

Thylakoid membranes retaining high oxygen-evolving activity (about 250 μmol O₂/mg Chl/h) were prepared from a marine centric diatom, *Chaetoceros gracilis*, after disruption of the cells by freeze–thawing. We also succeeded in purification of Photosystem II (PSII) particles by differential centrifugation of the thylakoid membranes after treatment with 1% Triton X-100. The diatom PSII particles showed an oxygen-evolving activity of 850 and 1045 μmol O₂/mg Chl/h in the absence and presence of CaCl₂, respectively. The PSII particles contained fucoxanthin chlorophyll a/c-binding proteins in addition to main intrinsic proteins of CP47, CP43, D2, D1, cytochrome b559, and the antenna size was estimated to be 229 Chl a per 2 molecules of pheophytin. Five extrinsic proteins were stoichiometrically released from the diatom PSII particles by alkaline Tris-treatment. Among these five extrinsic proteins, four proteins were red algal-type extrinsic proteins, namely, PsbO, PsbQ’, PsbV and PsbU, whereas the other one was a novel, hypothetical protein. This is the first report on isolation and characterization of diatom PSII particles that are highly active in oxygen evolution and retain the full set of extrinsic proteins including an unknown protein.

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Keywords: Diatom; Oxygen evolution; Photosystem II; Extrinsic protein; *Chaetoceros gracilis*

1. Introduction

Diatoms are eukaryotic, unicellular photosynthetic algae found throughout the world’s oceans and freshwater, and constitute one of the most important producers of the phytoplankton communities in aquatic ecosystems and the global carbon cycle. Photosynthesis by marine diatoms generates as much as 40% of the 45 to 50 billion metric tons of organic carbon produced each year in the sea [1], and their role in global carbon cycling is predicted to be comparable to that of all terrestrial rain forests combined [2]. The algae are among the most successful and diversified groups of photosynthetic eukaryotes, with probably over 100,000 extant species [3]. Their chloroplast was acquired by a secondary endosymbiosis event, i.e., the engulfment of a red alga by a eukaryotic host [4]. The thylakoid membranes of diatoms are arranged in stacks of three layers and are not segregated into granal and stromal lamellae as in green algae and plants. The pigment composition of diatoms differs from that of plants, green or red algae and cyanobacteria in that they

Abbreviations: CBB, Coomassie brilliant blue; Chl, chlorophyll; Cyt, cytochrome; DCBQ, 2,6-dichloro-p-benzoquinone; DCMU, dichlorophenyldimethylurea; DM, n-dodecyl-β-D-maltoside; FCP, fucoxanthin chlorophyll a/c-binding protein; HPLC, high-performance liquid chromatography; HTG, n-heptyl-β-D-thioglucoside; Mes, 2-morpholinoethanesulfonic acid; OG, n-octyl-β-D-glucoside; PBQ, phenyl-p-benzoquinone; PMSF, phenylmethyl sulfonyl fluoride; PSI and PSII, Photosystem I and Photosystem II; PVDF, polyvinylidene fluoride; SM, sucrose monolaurate; TMBZ, 3,3′,5,5′-tetramethylbenzidine

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contain chlorophyll (Chl) c, fucoxanthin and diadinoxanthin. Fucoxanthin Chl a/c-binding proteins (FCP; light-harvesting Chl proteins) have been purified and characterized from the centric diatom Cyclotella meneghiniana [5]. It is also known that diatoms have developed efficient photoprotective mechanisms in order to minimize photoinhibition that could result from their periodic exposure to excess light intensities when transported to the water column surface [6–8], and that they may perform C4 photosynthesis [9]. Furthermore, the complete nuclear, mitochondrial and plastid genome sequences of two diatoms Thalassiosira pseudonana and Phaeodactylum tricornutum were reported [10,11].

In spite of their significance, little is known about Photosystem II (PSII) in diatoms or other chromophytic Chl a/c containing algae. PSII is a thylakoid membrane-located, multiprotein-pigment complex which drives the light-induced electron transfer from water to plastoquinone with the concomitant production of molecular oxygen. The PSII complex contains a number of intrinsic proteins and 3–4 extrinsic proteins associated with the lumenal side. So far PSII particles and core complexes that are highly active in oxygen evolution and retain all of the extrinsic proteins have been isolated from cyanobacteria [12–14], red alga [15,16], Euglena [17], green alga [18] and higher plants [19,20]. Among these PSII complexes from a wide variety of organisms, the major intrinsic core proteins are largely conserved, whereas the extrinsic proteins which form the oxygen-evolving center of PSII are significantly different among different plant species. Among the extrinsic proteins, the 33 kDa protein (PsbO) which plays an important role in maintaining the stability and activity of the Mn-cluster is present in all of the oxygenic photosynthetic organisms. In contrast, the other extrinsic proteins that function to optimize the availability of Ca2+ and Cl− cofactors for water oxidation are different among different plant species. Cyanobacterial and red algal PSII complexes contain cytochrome (Cyt) c550 (PsbV) and the 12 kDa protein (PsbU) [12–16]. In red algal PSII, the three extrinsic proteins homologous to those of cyanobacteria are retained, but a fourth extrinsic protein, the unique 20 kDa protein which is required for the effective binding of PsbV and PsbU was newly found [16]. The 20 kDa protein had some similarities to the PsbQ of green algae in their amino acid sequences; based on this the 20 kDa protein was named PsbQ’ [21]. In contrast, Euglena, green algal and higher plant PSII complexes contain the 23 kDa (PsbP) and 17 kDa (PsbQ) proteins instead of PsbV and PsbU [17–20]. PsbP- and PsbQ-like proteins were also found in cyanobacterial PSII [14], and they have been suggested to be regulatory proteins necessary for the maintenance of optimally active PSII in nutrient-limiting media depleted of Cl−, Ca2+ or iron in the prokaryotic cyanobacteria [22,23].

The PsbV and PsbU proteins in cyanobacterial and red algal PSIIIs showed some similar functions as those of the PsbP and PsbQ proteins in green algal and higher plant PSII [12,16,24]. Recently, we examined the distribution of PSII extrinsic proteins in various oxygenic photosynthetic organisms using antibodies raised against extrinsic proteins from different sources, and showed that the extrinsic proteins had been diverged into cyanobacterial-type (PsbO, PsbV and PsbU), red algal-type (PsbO, PsbQ’, PsbV and PsbU), and green algal-type (PsbO, PsbP and PsbQ) during early phases of evolution after a primary endosymbiosis [25]. We also showed that chromophytic Chl a/c containing algae such as diatoms and brown algae, which resulted from red algal secondary endosymbiosis, contained the red algal-type extrinsic proteins [25]. In order to further elucidate the extrinsic proteins in chromophytic Chl a/c containing algae, PSII that is highly active in oxygen evolution and retains the extrinsic proteins needs to be isolated from these algal cells. There are, however, no reports relating to the isolation and characterization of chromophytic Chl a/c containing algal PSII and their extrinsic proteins.

As described by Martinson et al. [26], detailed studies of diatom PSII have been hampered at the level of obtaining thylakoid membranes that are capable of oxygen evolution, and a major stumbling block in working with these algae has been the difficulty in breaking the silica frustule surrounding the diatom cell without damaging intracellular structures. They reported that diatom thylakoid membranes purified in 2 M sorbitol using the protoplast/French press method showed the best preservation of in vivo fluorescence emission signals, and that the PSII activity with ferricyanide was completely inhibited by DCMU. The rates of electron transport in the purified thylakoid membranes were, however, significantly lower than the in vivo rates (about 50 μmol O2/mg Chl/h).

In this study, we found that the cells from a marine centric diatom, Chaetoceros gracilis, were readily disrupted by freeze–thawing without using mechanical treatments of sonication or French press, and that the thylakoid membranes prepared by freeze–thawing showed high oxygen-evolving activity, as described by Ikeda et al. [27]. We also succeeded in isolation of PSII particles from C. gracilis that were highly active in oxygen evolution and retained the extrinsic proteins by a simple method using differential centrifugation of Triton-treated thylakoid membranes. Five extrinsic proteins were found to be present in the PSII particles by alkaline Triton-treatment; all of them were identified by immunoblotting analysis using antibodies against various extrinsic proteins from different plant species and by N-terminal amino acid sequencing.

2. Materials and methods

2.1. Cultures

A marine centric diatom, C. gracilis Schütt (UTEX LB 2658), was grown in 8 l of artificial seawater (SEA LIFE) medium containing 1/1000 volume of KW21 (marine algae culture medium from Daichi Seimo Co., Ltd., Kumamoto, Japan) and 0.18 mM sodium metasilicate, at 25 °C under continuous illumination at 30–35 μmol photon m−2 s−1 and air bubbling. The cells were grown for about 10 days (early stationary stage), harvested by using PELLICON (Millipore, Billerica, MA) and then collected at 3000 ×g for 3 min.

2.2. Disruption of the cells from C. gracilis by freeze–thawing

The C. gracilis cells of about 12 g wet weight were suspended in 32 ml of a medium containing 1 M betaine, 50 mM 2-morpholinoethanesulfonic acid (Mes) (pH 6.5) and 5 mM MgCl2 (about 1.8 mg Chl/ml) and then frozen in liquid N2 for 10 min. The frozen cells were quickly thawed at room temperature
within 10 min. This simple freeze–thawing treatment disrupted the *C. gracilis* cells completely.

### 2.3. SDS-PAGE, immunological assays and heme-staining

Samples were solubilized with 5% lithium lauryl sulfate and 75 mM dithiothreitol. The solubilized samples (15 μg Chl for thylakoid membranes, Fractions 1 and 5; 10 μg Chl for Fractions 2, 3 and 4) were applied to an SDS-polyacrylamide gel containing a gradient of 12–18% acrylamide and 7.5 M urea. For separation of PsbU and an intrinsic protein, a gradient gel of 16–22% acrylamide and 7.5 M urea was used [28].

For Western blotting, proteins on the gel were transferred onto a polyvinylidene fluoride (PVDF) membrane, reacted with respective antibodies and visualized with 4-chloro-1-naphthol after incubation with biotinylated anti-rabbit IgG and peroxidase-conjugated streptavidin. Antibodies against FCP from the raphidophyte *Heterosigma akashiwo* and PsbU from *Thermosynechococcus vulcanus* were kindly provided by Profs. B. R. Green and J.-R. Shen, respectively. The other antibodies were prepared as described in Enami et al. [25].

Heme-staining was carried out with TMBZ/H2O2 on the gel, according to the procedure described in Thomas et al. [29] and Shen and Inoue [12].

### 2.4. Absorption spectra

Absorption spectra were measured using a DU 800 UV/visible spectrophotometer (Beckman Coulter, Inc.) at room temperature and adjusted to the same level of absorption at the α band of Chl α for thylakoids and PSII particles, respectively.

### 2.5. Assay of oxygen evolution

Oxygen evolution was measured with a Clark-type oxygen electrode at 25 °C with 0.4 mM phenyl-p-benzoquinone (PBQ), 0.5 mM 2,6-dichloro-p-benzoquinone (DCBQ) or 2 mM ferricyanide as electron acceptors. The measurements were carried out in a medium of 0.4 M sucrose and 40 mM Mes (pH 6.5) in the absence and presence of 5 mM CaCl2. Chl concentrations were determined by the Edman degradation method with a Procise HT protein sequencing system (Applied Biosystems, Foster City, CA).

### 2.6. Dissociation of extrinsic proteins

The diatom PSII particles were treated with 1 M Tris (pH 8.5) at 0.5 mg Chl/ml for 30 min at 0 °C in the dark. The samples were centrifuged at 40,000 × g for 20 min after addition of 10% polyethylene glycol 6000. The polypeptides of the resulting precipitates and supernatants were analyzed by SDS-PAGE.

### 2.7. Determination of N-terminal amino acid sequences

The extrinsic proteins were released by alkaline Tris-treatment from the diatom PSII particles, separated by electrophoresis, and the proteins on the gel were transferred onto a PVDF membrane, stained with 0.1% Coomassie brilliant blue (CBB) G-250 and destained with 40% methanol, 10% acetic acid. Each band on the membrane was cut out, and their N-terminal sequences were determined in 90% acetone using the equation of Jeffrey and Humphrey [30].

### 2.8. Analysis of pigments and plastoquinone

The amounts of Chl a, Chl c, fucoxanthin, diadinoxanthin, β-carotene, pheophytin and plastoquinone in the diatom PSII particles were determined by reverse-phase HPLC with a Prodigy 5 (ODS 3,100Å) column (150×4.60 mm) (Phenomenex Inc., Torrance, CA) equipped to a Shimadzu LC-10 AD system with a SCL-10A controller, as described previously [31,32].

### 2.9. Gel filtration column chromatography

Gel filtration column chromatography was carried out using a Sephadex S-300 (GE Healthcare, Buckinghamshire, England). The running buffer contained 25% glycerol, 10 mM MgCl2, 5 mM CaCl2 and 0.04% n-dodecyl-β-D-maltoside (DM) and a flow rate of 0.5 ml/min was used.

### 3. Results

#### 3.1. Disruption of the cells and preparation of thylakoid membranes from *C. gracilis*

We first attempted to disrupt the *C. gracilis* cells by agitation with glass beads or an ultrasonic disruptor to break their silica-based rigid cell wall. These treatments, however, completely abolished the oxygen-evolving activity. In various attempts, we found that the *C. gracilis* cells were readily disrupted by one cycle of freeze–thawing in a medium containing 1 M betaine, 50 mM Mes (pH 6.5) and 5 mM MgCl2. The cell suspensions after this disruption were highly active in oxygen evolution, as described by Ikeda et al. [27]. The cells disrupted by freeze–thawing were incubated in the presence of DNase I and 1 mM PMSF for 30 min at 0 °C in the dark to degrade DNA in the cell suspensions. The supernatants after centrifugation at 3000 × g for 3 min were centrifuged at 40,000 × g for 10 min and its precipitates (thylakoid membranes) were suspended in a medium containing 1 M betaine and 50 mM Mes (pH 6.5) (buffer A) (see Fig. 1).

Table 1 (upper part) shows oxygen-evolving activities of the thylakoid membranes isolated from *C. gracilis* in the absence and presence of 5 mM CaCl2 using various electron acceptors. The activity of thylakoid membranes in the absence of CaCl2 was 243–265 μmol O2/mg Chl/h with PBQ as electron acceptor, while the activity decreased to 150–192 μmol O2/mg Chl/h with DCBQ as the acceptor, and further decreased to 27–41 μmol O2/mg Chl/h with ferricyanide as the acceptor. These activities were comparable to those of intact cells (data not shown). The oxygen-evolving activity of thylakoid membranes was slightly
stimulated by addition of 5 mM CaCl$_2$. For example, the activity with PBQ was stimulated to 304–322 $\mu$mol O$_2$/mg Chl/h (about 1.2-fold) by addition of 5 mM CaCl$_2$. In addition, the activity with PBQ was completely inhibited by 10 $\mu$M DCMU.

### 3.2. Preparation of PSII particles from C. gracilis

As we succeeded in preparation of highly active thylakoid membranes from *C. gracilis*, we attempted to isolate PSII particles using the thylakoid membranes. Before preparation of PSII particles, we examined the effects of various detergents on solubilization of the thylakoid membranes and their oxygen-evolving activity. The thylakoid membranes were treated with various detergents at a concentration of 1% in buffer A, at 1 mg Chl/ml for 5 min at 0 °C in the dark. The treated membranes were centrifuged at 40,000 $\times$ g for 10 min to remove unsolubilized materials (Fraction 1). About 14% of Chl was distributed in Fraction 1, indicating that about 86% of Chl were associated with the proteins solubilized by the Triton-treatment (Table 3). These supernatants were centrifuged at 50,000 $\times$ g for 20 min, and the supernatants were suspended in buffer A and designated Fraction 2. Fraction 2 was green in color and its Chl $a/c$ ratio (w/w) was about 7.4 (Table 3), suggesting that this fraction contains little Chl c. The yield of Fraction 2 was about 12% based on Chl contents, and the fraction showed little oxygen-evolving activity (Table 3). As shown in lane 3 of Fig. 2, Fraction 2 consisted of a large subunit with an apparent molecular weight of around 60,000 and several small subunits below 25,000. The large subunit band (asterisk in Fig. 2) crossreacted with the antibody raised against the PSI large subunits (PsaA/B) (data not shown). These indicated that Fraction 2 is mainly composed of Photosystem I (PSI). The supernatants after centrifugation at 50,000 $\times$ g for 20 min were centrifuged at 146,000 $\times$ g for 20 min and the pellets were suspended in buffer A (Fraction 3). Fraction 3 contained residual PSI not precipitated by centrifugation at 50,000 $\times$ g for 20 min and slight PSII (lane 4 in Fig. 2) and its yield was about 2% based on Chl contents (Table 3). This procedure was needed to precipitate the residual PSI completely. The supernatants after centrifugation at 146,000 $\times$ g for 20 min were centrifuged at 40,000 $\times$ g for 10 min after addition of 10% polyethylene glycol 6000, and the resulting precipitates were suspended in buffer A, and their Chl contents and oxygen-evolving activities were measured. As shown in Table 2, about 80% of the thylakoid membranes based on Chl contents were effectively solubilized by 1% Triton X-100, n-dodecyl-$\beta$-D-maltoside (DM), n-heptyl-$\beta$-D-thiogluco-side (HTG), n-octyl-$\beta$-D-gluco-side (OG) and sucrose monolaurate (SM). Among 5 detergents tested, the Triton-extracts showed the oxygen-evolving activity comparable to that of the thylakoid membranes, while the activity of the DM- and OG-extracts considerably decreased and that of the HTG- and SM-extracts further decreased. Thus, Triton X-100 was chosen as the detergent for solubilization of the thylakoid membranes in this study.

As shown in Fig. 1, the thylakoid membranes treated with 1% Triton X-100 in buffer A at 1 mg Chl/ml for 5 min at 0 °C were fractionated by differential centrifugation. The treated thylakoid membranes were centrifuged at 40,000 $\times$ g for 10 min to remove unsolubilized materials (Fraction 1). About 14% of Chl was distributed in Fraction 1, indicating that about 86% of Chl were associated with the proteins solubilized by the Triton-treatment (Table 3). The resultant supernatants were centrifuged at 50,000 $\times$ g for 20 min, and the supernatants were suspended in buffer A and designated Fraction 2. Fraction 2 was green in color and its Chl $a/c$ ratio (w/w) was about 7.4 (Table 3), suggesting that this fraction contains little Chl c. The yield of Fraction 2 was about 12% based on Chl contents, and the fraction showed little oxygen-evolving activity (Table 3). As shown in lane 3 of Fig. 2, Fraction 2 consisted of a large subunit with an apparent molecular weight of around 60,000 and several small subunits below 25,000. The large subunit band (asterisk in Fig. 2) crossreacted with the antibody raised against the PSI large subunits (PsaA/B) (data not shown). These indicated that Fraction 2 is mainly composed of Photosystem I (PSI). The supernatants after centrifugation at 50,000 $\times$ g for 20 min were centrifuged at 146,000 $\times$ g for 20 min and the pellets were suspended in buffer A (Fraction 3). Fraction 3 contained residual PSI not precipitated by centrifugation at 50,000 $\times$ g for 20 min and slight PSII (lane 4 in Fig. 2) and its yield was about 2% based on Chl contents (Table 3). This procedure was needed to precipitate the residual PSI completely. The supernatants after centrifugation at 146,000 $\times$ g for 20 min were centrifuged at 40,000 $\times$ g for 10 min after addition of 10% polyethylene glycol 6000, and the precipitates were suspended in the same buffer (Fraction 4). Fraction 4 was brown in color and its Chl $a/c$ ratio was about 2.5 (Table 3), similar to those of thylakoid membranes. The yield of Fraction 4 was about 18% based on Chl contents (Table 3). Fraction 4 showed a high oxygen-evolving activity of 750–950 $\mu$mol O$_2$/mg Chl/h in the

### Table 1

<table>
<thead>
<tr>
<th>Detergents</th>
<th>Oxygen evolution ((\mu)mol O$_2$/mg Chl/h)</th>
<th>Yield of Chl solubilized by detergents</th>
<th>Chl $a/c$ ratio</th>
<th>Oxygen evolution ((\mu)mol O$_2$/mg Chl/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-treatment</td>
<td>250±12</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triton X-100</td>
<td>233±11</td>
<td>78±5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>150±25</td>
<td>82±5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HTG</td>
<td>110±11</td>
<td>74±6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OG</td>
<td>142±8</td>
<td>77±5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM</td>
<td>125±21</td>
<td>86±7</td>
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<td></td>
</tr>
</tbody>
</table>

a Values shown are average of three measurements.
b Oxygen evolution was measured in 0.4 M sucrose and 40 mM (pH 6.5) with PBQ as electron acceptor in the absence of CaCl$_2$.

c Oxygen evolution was measured in 0.4 M sucrose and 40 mM Mes (pH 6.5) with PBQ as electron acceptor in the absence of CaCl$_2$.

d Yield of total oxygen-evolving activity, calculated from oxygen evolution multiplied by the Chl content.
absence of CaCl₂, and the yield was about 62% based on total oxygen-evolving activity (Table 3), indicating that PSII was recovered to Fraction 4 at a high yield. The supernatants after the final centrifugation (Fraction 5) mainly contained polypeptide bands that crossreacted with the antibody against FCP from the raphidophyte Heterosigma akashiwo (open circle in lane 6 of Fig. 2) (data not shown), and its yield was about 39% based on Chl contents (Table 3). Each fraction was stored at −196 °C until use.

3.3. Characterization of the PSII particles from C. gracilis

3.3.1. Oxygen-evolving activity

Table 1(lower part) shows oxygen-evolving activities of the isolated PSII particles (Fraction 4 in Fig. 1 and Table 3) in the absence of CaCl₂ using various electron acceptors. The activity of PSII particles in the absence of CaCl₂ was 752–948 μmol O₂/mg Chl/h with PBQ as the electron acceptor, while the activity decreased to 405–583 μmol O₂/mg Chl/h with DCBQ as the acceptor, and decreased further to 66–82 μmol O₂/mg Chl/h with ferricyanide as the acceptor. These activities were slightly stimulated by the addition of 5 mM CaCl₂. For example, the activity with PBQ was stimulated to 894–1196 μmol O₂/mg Chl/h (about 1.2-fold) by the addition of 5 mM CaCl₂. The activity with PBQ was completely inhibited by 10 μM DCMU. These properties of oxygen evolution in the PSII particles were largely similar to those of thylakoid membranes, indicating that the PSII particles have preserved their intactness after isolation.

3.3.2. Absorption spectrum

The absorption spectrum of the isolated PSII particles was compared with that of the thylakoid membranes. As shown in Fig. 3, the PSII particles showed absorption maxima at 439 and 672 nm arising from Chl a, and shoulders at 465 and 635 nm contributed by Chl c, which were similar to those of thylakoid membranes. In contrast, the absorbance between 475 and 565 nm due to fucoxanthin and diadinoxanthin decreased in the PSII particles compared with that in thylakoid membranes, indicating that these pigments were partially removed in the isolated PSII particles.

3.3.3. Pigments and plastoquinone composition

Table 4 shows the amounts of various pigments and plastoquinone in the PSII particles (first line). On the basis of two molecules of pheophytin a, 229 molecules of Chl a, 86 molecules of Chl c, 162 molecules of fucoxanthin, 13 molecules of diadinoxanthin, 16 molecules of β-carotene and 5 molecules of plastoquinone were contained in the PSII particles. Thus, the antenna size of the PSII particles was estimated to be 229 Chl a per 2 molecules of pheophytin. The PSII particles contained about 5 plastoquinone per 2 molecules of pheophytin. This suggests that PSII prepared in this study is a type of PSII membrane particles in which extra plastoquinones are contained.

3.3.4. Identification of PSII subunits

In order to identify the protein components in the PSII particles, immunoblotting analysis was carried out with antibodies raised against major intrinsic proteins and PsbO in Table 4.

<table>
<thead>
<tr>
<th>Pigments (molar ratio to 2 Phe a)*</th>
<th>Chl c1+c2</th>
<th>Fx</th>
<th>Dx</th>
<th>Chl a</th>
<th>β-Car</th>
<th>PQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS II</td>
<td>86.1</td>
<td>162</td>
<td>13.2</td>
<td>229</td>
<td>15.5</td>
<td>5.25</td>
</tr>
<tr>
<td>Fraction 1</td>
<td>52.6</td>
<td>90.4</td>
<td>8.33</td>
<td>169</td>
<td>14.3</td>
<td>4.31</td>
</tr>
<tr>
<td>Fraction 2</td>
<td>51.3</td>
<td>89.6</td>
<td>7.17</td>
<td>167</td>
<td>14.2</td>
<td>4.28</td>
</tr>
</tbody>
</table>

Fx, fucoxanthin; Dx, diadinoxanthin; β-Car, β-carotene; PQ, plastoquinone.

* Values are expressed on the basis of 2 molecules of pheophytin a.
spinach PSII and FCP from the raphidophyte *Heterosigma akashiwo*. As shown in Fig. 4, antibodies against spinach CP47, CP43, PsbO, D2, D1 and the α subunit of Cyt b559 crossreacted with the corresponding components in the PSII particles, confirming the presence of these homologous components in the PSII particles of *C. gracilis*. Furthermore, four bands around 20 kDa were determined to be FCP subunits.

### 3.3.5. Dissociation of the extrinsic proteins by alkaline Tris-treatment

In order to determine the extrinsic proteins in *C. gracilis* PSII, we analyzed the proteins released by alkaline Tris-treatment of the PSII particles. All of the extrinsic proteins in PSII membrane particles and PSII core complexes from a wide variety of organisms (spinach [33], green alga [18], *Euglena* [17], red alga [15, 16], cyanobacteria [12]) have been reported to be released by alkaline Tris-treatment. Fig. 5 shows the extrinsic proteins in the diatom PSII particles released by 1 M Tris (pH 8.5). Five proteins were released by the Tris-treatment (shown as bands a–e in Fig. 5). When SDS-PAGE was performed with a gradient gel containing 12–18% acrylamide and 6 M urea, band e was co-migrated with an intrinsic protein (lanes 2 and 3 in Fig. 5-A). Band e and the intrinsic protein band were clearly separated in SDS-PAGE with a gradient gel containing 16–22% acrylamide and 7.5 M urea, as shown in lanes 2 and 3 of Fig. 5-B. These indicate that the five extrinsic proteins were completely released by the Tris-treatment. Two faint bands, one migrated above CP47 and the other one migrated below band c (asterisk in lane 3 in Fig. 5-A and -B) were identified to be a large subunit and a small subunit of RubisCO, respectively, from their N-terminal amino acid sequences (data not shown).

### 3.3.6. Identification of the extrinsic proteins in *C. gracilis* PSII

In order to identify the five extrinsic proteins in the diatom PSII particles, immunoblotting analysis was carried out with antibodies raised against various extrinsic proteins from higher plant (*Spinacia oleracea*) (PsbO, PsbP and PsbQ), green alga (*Chlamydomonas reinhardtii*) (PsbQ), red alga (*Cyanidium caldarium*) (PsbQ’, PsbV and PsbU) and cyanobacterium (*Thermosynechococcus vulcanus*) (PsbU), as the antibodies against PsbO, PsbP and PsbV can be used as common antibodies for these proteins among different species but the other antibodies have a high species-specificity and cannot be used as common antibodies to detect the presence of these proteins among different species [25]. As shown in Fig. 6-A, the bands a, b and c among the five extrinsic proteins released from the diatom PSII particles by the Tris-treatment crossreacted with antibodies against spinach PsbO (lane 3), red algal PsbQ’ (lane 7) and red algal PsbV (lane 8), while the bands d and e did not crossreact with any antibodies. Antibodies against spinach PsbP (lane 4), spinach PsbQ (lane 5) and green algal PsbQ (lane 6) did not crossreact with any subunits in the diatom PSII particles, suggesting that the diatom PSII contains no green algal- and higher plant-type extrinsic proteins, PsbP and PsbQ. Based on homology search with the two available genomes from *T. pseudonana* and *P. tricornutum* ([http://genome.jgi-psf.org/Thaps3/Thaps3.home.html](http://genome.jgi-psf.org/Thaps3/Thaps3.home.html) PIs. link, [http://genome.jgi-psf.org/Phatr2/Phatr2.home.html](http://genome.jgi-psf.org/Phatr2/Phatr2.home.html)), the homologue gene coding for PsbP-like proteins rather than green algal- and higher plant-type extrinsic PsbP were found in the two diatoms. Recently, it has been reported that higher plants have two nuclear-encoded genes for PsbP homologs (PsbP-like proteins: PPLs) that show significant sequence similarity to a cyanobacterial PsbP homolog (cyanoP) in addition to the extrinsic PsbP protein [34].

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**Fig. 4.** Identification of PSII components of *Chaetoceros gracilis* by immunoblotting analysis. Lane C, CBB-staining of the PSII particles; lane 1, anti-CP47; lane 2, anti-CP43; lane 3, anti-PsbO; lane 4, anti-D2; lane 5, anti-D1; lane 6, anti-Cyt b559 (α); lane 7, anti-FCP.

**Fig. 5.** Dissociation of extrinsic proteins from the PSII particles of *Chaetoceros gracilis* by treatment with 1 M alkaline Tris (pH 8.5). (A) SDS-PAGE with a gradient gel of 12–18% acrylamide and 6 M urea. (B) SDS-PAGE with a gradient gel of 16–22% acrylamide and 7.5 M urea. Lane 1, PSII (control); lane 2, precipitate after alkaline Tris-treatment; lane 3, supernatant after alkaline Tris-treatment. Asterisks are the bands which were identified as large and small subunits of RubisCO, respectively.
These suggest that diatoms have the PsbP-like proteins but not green algal- and higher plant-type extrinsic PsbP.

The presence of PsbV in the diatom PSII particles was also confirmed by heme-staining of the gel, which permitted detection of c-type cytochromes on the gel [12,15]. As shown in Fig. 6-B, three c-type cytochromes, which were estimated to be Cyt f, Cyt c550 (PsbV) and Cyt c553 from their apparent molecular weights, were detected in the thylakoid membranes by the heme-staining method (lane 1). Of these cytochromes, only PsbV was detected in the PSII particles (lane 3), suggesting that no Cyt b/f complexes are contaminated in the isolated PSII particles. No c-type cytochromes were found in the PSI particles (lane 2), and Cyt f and Cyt c553 were detected in the supernatant (Fraction 5) after the final centrifugation (lane 4).

The antibody against cyanobacterial or red algal PsbU did not crossreact with any extrinsic proteins in the diatom PSII particles (lanes 9 and 10 in Fig. 6-A). This is probably due to the high species-specificity of the PsbU antibody, as described in Enami et al. [25]. To identify the extrinsic proteins of bands d and e, N-terminal amino acid sequences of these extrinsic proteins were determined. Table 5 shows N-terminal amino acid sequences of the two extrinsic proteins together with those of the other three extrinsic proteins. Based on homology search with available diatom EST database (http://avesthagen.sznbowler.com) [35], bands a, b, c, d and e were identified to be PsbO, PsbQ', PsbV, a novel, hypothetical protein previously unknown and PsbU, respectively (Table 5).

### Table 5

Identification of the extrinsic proteins in PSII of *Chaetoceros gracilis* by their N-terminal amino acid sequences

<table>
<thead>
<tr>
<th>Bands</th>
<th>N-terminal amino acid sequences</th>
<th>Identification from database search</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>LTKSQINELSYLOVKGTVGLA</td>
<td>PsbO</td>
</tr>
<tr>
<td>b</td>
<td>AVGESPRSFVGLVVDGTYSK</td>
<td>PsbQ'</td>
</tr>
<tr>
<td>c</td>
<td>IDLDHEATRTVVVDNAGTTVV</td>
<td>PsbV</td>
</tr>
<tr>
<td>d</td>
<td>DGAIVSSATRARGLYGVHR</td>
<td>Novel, unknown protein</td>
</tr>
<tr>
<td>e</td>
<td>VIDYENIGYLGGSIVDNN</td>
<td>PsbU</td>
</tr>
</tbody>
</table>

3.3.7. Polypeptide and pigment compositions of *C. gracilis* PSII after gel filtration column chromatography

As described above, the diatom PSII prepared in this study contained RubisCO subunits. In order to confirm whether the subunits are contaminations in the PSII particles, the PSII particles were subjected to gel filtration column chromatography using Sephadex S-300. The elution profile and polypeptide patterns of the typical fractions obtained were shown in Fig. 7-A and -B, respectively. The molecular size of the main peak corresponds to about 800 kDa. Fraction 1 around the main peak and Fraction 2 around the shoulder appeared in the elution pattern of Fig. 7-A were composed of PSII components including the five extrinsic proteins but not RubisCO subunits, while Fraction 3 around the minor peak was mainly consisted of FCP. Note that the molecular weights of FCP bands in Fraction 3 are different from those of FCP in Fractions 1 and 2 (Fig. 7-B). These results indicate that FCP in Fraction 3 and RubisCO are just co-precipitated with the PSII particles. In contrast, the five extrinsic proteins were not released by the gel filtration, indicating that the novel, previously unknown extrinsic protein is truly a component of the diatom PSII together with the other four extrinsic proteins, which tightly associate with the PSII particles.

The amounts of RubisCO subunits in the diatom PSII particles varied from preparation to preparation. In fact, the PSII particles used in the gel filtration contained little RubisCO subunits as shown in lane P of Fig. 7-B, while the PSII particles shown in Figs. 2, 4, 5 and 6 contained a large amount of...
Rubisco subunits. When the PSII particles containing a large amount of Rubisco subunits were applied to the gel filtration, the Rubisco subunits were eluted at a longer elution time compared to PSII particles (data not shown). This also indicates that the Rubisco subunits are just contaminations in the PSII particles, whereas the novel, previously unknown hypothetical protein is a component of diatom PSII.

The amounts of various pigments and plastoquinone in Fractions 1 and 2 obtained by the gel filtration were also analyzed. As shown in Table 4, the values of these components were nearly equal between Fractions 1 and 2. On the basis of two molecules of pheophytin $\alpha$, 167–169 molecules of Chl $\alpha$, 51–53 molecules of Chl $c$, 90 molecules of fucoxanthin, 7–8 molecules of diadinoxanthin, 14 molecules of $\beta$-carotene and 4 molecules of plastoquinone were contained in Fractions 1 and 2. The amounts of pigments in Fractions 1 and 2 decreased compared with those of the PSII particles before the gel filtration. The difference of the number of pigments between the initial and gel-filtrated PS II particles is attributable to FCP that was eluted separately from PS II particles (Fraction 3), suggesting that these FCPs were associated with PSII loosely. Fractions 1 and 2 contained about 4 plastoquinones per 2 molecules of pheophytin even after the gel filtration, which is larger by 2 molecules compared to the number of bound plastoquinone in the crystallographic model of PS II [36,37]. The extra plastoquinones detected in these Fractions are probably the plastoquinone included in the membranes of these Fractions.

4. Discussion

In this study, we succeeded for the first time in the preparation of PSII particles retaining a high oxygen-evolving activity and all of the extrinsic proteins from a chromophytic alga. The success is largely due to the finding that the cells of C. gracilis can be readily disrupted by a simple freeze–thawing method, as described recently by Ikeda et al. [27]. Treatments by sonication, French press or glass beads usually used for disruption of various algal cells [12,15,17,18] completely inactivated the oxygen evolution in the case of C. gracilis. So far, studies on diatom PSII have been hindered at the level of obtaining thylakoid membranes that are capable of oxygen evolution, and a major stumbling block in working with these algae has been the difficult nature of breaking silica-based rigid cell wall without damaging intracellular structures. The finding of the disruption of diatom cells by the simple freeze–thawing method will greatly benefit biochemical studies on photosynthesis of diatoms. However, the simple freeze–thawing method is not effective to all of the diatom species. In our preliminary experiments, the freeze–thawing method readily disrupted the cells of a centric diatom, T. pseudonana, but not a pennate diatom, P. tricornutum. Previously, Gugliemelli et al. [38] also reported that the cells of P. tricornutum could not be disrupted by several cycles of freeze–thawing treatment.

The other important point for preparing the thylakoid membranes retaining high oxygen-evolving activities from C. gracilis is to culture the diatom under a low light intensity at about 30–35 $\mu$mol photon m$^{-2}$ s$^{-1}$. When the diatom was grown under high light intensity above 800 $\mu$mol photon m$^{-2}$ s$^{-1}$, the thylakoid membranes prepared by the same method showed only little oxygen-evolving activity (data not shown). Diatoms living in natural waters experience large fluctuations in light intensity due to unpredictable water motions that can vary over several orders of magnitude [8,31]. Therefore, diatoms have developed efficient photosynthesis under weak light by increasing the antenna size and effective photoprotective mechanisms against strong light by diadinoxanthin cycle [6–8,31]. The light adaptation mechanisms developed in diatom cells may be one of the reasons why diatom thylakoid membranes retaining high oxygen-evolving activities were prepared from the diatom cultured under a low light intensity but not under high light intensity.

![Fig. 7. Elution pattern from gel filtration column chromatography of the PSII particles of Chaetoceros gracilis (A) and polypeptide profiles of typical fractions obtained by the chromatography (B).](image_url)
The PSII particles isolated from *C. gracilis* in this study contained considerable amounts of FCP and extra plastoquinone (Figs. 3 and 4, Table 4), which indicates that the preparation is similar to the BBY-type PSII membrane particles prepared from spinach [19]. In fact, the isolated PSII particles were brown in color and its Chl a/c ratio was about 2.5 (w/w) (Table 3), and their antenna size was estimated to be 229 Chl a per 2 molecules of phophytin (Table 4), which was more than 4-fold larger than those of cyanobacterial and red algal PSII complexes [12,15]. The oxygen-evolving activity of the PSII particles prepared in this study (about 850 μmol O₂/mg Chl/h) was lower than those of cyanobacterial and red algal PSII complexes (usually above 2500 μmol O₂/mg Chl/h) [12,15]. This can be ascribed to the large antenna size of the PSII particles due to the presence of a considerable amount of FCP. The high amount of FCP leads to the large molecular size of PS II particles as large as ~800 kDa, which agrees with an early report that a manganese–copper–pigment–protein complex isolated from *P. tricornutum* had a molecular weight of 850 kDa [39]. Since all of the crystal structures of cyanobacterial PSII core [36,37,40] are reported to be a dimer with an apparent molecular mass of ~580 kDa [41], the present results suggest that the diatom PSII exists as a dimer also.

Five extrinsic proteins were released by alkaline Tris-treatment of the diatom PSII particles (Fig. 5). These extrinsic proteins were identified to be PsbO, PsbQ', PsbV, a novel hypothetical protein and PsbU (Fig. 6 and Table 5). These indicate that the diatom PSII contains the red algal-type extrinsic proteins, in agreement with our previous proposal that chromophytic Chl a/c containing algae such as diatoms and brown algae which resulted from red algal secondary endosymbiosis contain the red algal-type extrinsic proteins [25]. A remarkable feature of the diatom PSII particles is that its PSII contains a novel, previously unknown extrinsic protein in addition to the four proteins of PsbO, PsbQ', PsbV and PsbU well characterized in other organisms. The novel extrinsic protein was stoichiometrically released by alkaline Tris-treatment of the PSII particles together with the other four extrinsic proteins (Fig. 5). Furthermore, the novel extrinsic protein was tightly associated with PSII even after gel filtration together with the other four extrinsic proteins (Fig. 7) and specifically bound to PSII but not PSI (data not shown). These suggest that the novel protein is one of the extrinsic proteins in the diatom PSII.

The protein composition of PSII isolated from *C. gracilis* was summarized in Fig. 8, which showed that the PSII particles contained over 19 main polypeptide bands. Among these subunits, we identified 16 polypeptide bands including 5 intrinsic proteins of CP47, CP43, D2, D1 and a large subunit of Cyt b559, 5 extrinsic proteins of PsbO, PsbQ', PsbV, PsbU and a novel, hypothetical protein, 4 subunits of FCP and 2 subunits of RubisCO. Several polypeptide bands remain to be identified. We will further purify the diatom PSII and identify all of the protein components. Nevertheless, the present study represents the first successful isolation and characterization of PSII with a high oxygen-evolving activity from a diatom, which identified a novel extrinsic protein previously unknown in any other organisms.

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