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Purification and characterization of a stable oxygen-evolving Photosystem II complex from a marine centric diatom, *Chaetoceros gracilis*

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ABSTRACT

Oxygen-evolving Photosystem II particles (crude PSII) retaining a high oxygen-evolving activity have been prepared from a marine centric diatom, *Chaetoceros gracilis* (Nagao et al., 2007). The crude PSII, however, contained a large amount of fucoxanthin chlorophyll *a*/*c*-binding proteins (FCP). In this study, a purified PSII complex which was deprived of major components of FCP was isolated by one step of anion exchange chromatography from the crude PSII treated with Triton X-100. The purified PSII was still associated with the five extrinsic proteins of PsbO, PsbQ', PsbV, Psb31 and PsbU, and showed a high oxygen-evolving activity of 2135 μmol O_2 (mg Chl *a*)$^{-1}$ h^{-1} in the presence of phenyl-*p*-benzoquinone which was virtually independent of the addition of $CaCl_2$. This activity is more than 2.5-fold higher than the activity of the crude PSII. The activity was completely inhibited by 3-(3,4)-dichlorophenyl-(1,1)-dimethylurea (DCMU). The purified PSII contained 42 molecules of Chl *a*, 2 molecules of diadinoxanthin and 2 molecules of Chl *c* on the basis of two molecules of pheophytin *a*, and showed typical absorption and fluorescence spectra similar to those of purified PSIIs from the other organisms. In this study, we also found that the crude PSII was significantly labile, as a significant inactivation of oxygen evolution, chlorophyll bleaching and degradation of PSII subunits were observed during incubation at 25 °C in the dark. In contrast, these inactivation, bleaching and degradation were scarcely detected in the purified PSII. Thus, we succeeded for the first time in preparation of a stable PSII from diatom cells.

1. Introduction

Diatoms constitute one of the most important producers of the phytoplankton communities in aquatic ecosystems and play an important role in the global carbon cycle [1], with their contribution to the global carbon cycling predicted to be comparable to that of all terrestrial rain forests combined [2]. In spite of their significance, little is known about Photosystem II (PSII) in diatoms. As described by Martinson et al. [3], 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. Recently, we succeeded for the first time in preparation of thylakoid membranes and PSII particles (crude PSII) retaining a high oxygen-evolving activity from a marine centric diatom, *Chaetoceros gracilis* [4]. The success is largely due to the finding that the diatom cells are readily disrupted by a simple freeze–thawing method [5] without inactivation of the oxygen evolution. Treatments by sonication, French press or glass beads usually used for disruption of various algal cells [6–11] completely inactivated the oxygen evolution in the case of the diatom cells. Thus, the finding of disruption of diatom cells by the simple freeze–thawing method will greatly benefit biochemical studies on photosynthesis of diatoms.

The crude PSII prepared from *C. gracilis* contained five extrinsic proteins. Among these five proteins, four proteins were red algal-type extrinsic proteins of PsbO, PsbQ', PsbV and PsbU well characterized in cyanobacteria and red algae [6–8,11–14], whereas the fifth one was a novel protein referred to as Psb31 following the nomenclature for PSII

Abbreviations: Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4)-dichlorophenyl-(1,1)-dimethylurea; EDTA, ethylenediaminetetraacetic acid disodium salt; FCP, fucoxanthin chlorophyll *a*/*c*-binding proteins; MES, 2-morpholinoethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PBQ, phenyl-*p*-benzoquinone; PQ, plastoquinone; PSII, Photosystem II; Q_A, the first quinone acceptor of Photosystem II; Q_B, the second quinone acceptor of Photosystem II; RuBisCO, ribulose 1,5-bisphosphate carboxylase/oxygenase

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subunits [4,15]. The gene encoding the Psb31 protein was cloned and sequenced from *C. gracilis* [15]. The deduced sequence contained three characteristic leader sequences targeted for chloroplast endoplasmic reticulum membrane, chloroplast envelope membrane and thylakoid membrane, indicating that the novel Psb31 protein is encoded in the nuclear genome and constitutes one of the extrinsic proteins located on the lumenal side [15].

The crude PSII, however, contained a large amount of fucoxanthin chlorophyll *a*/*c*-binding proteins (FCP). In this study, we attempted to remove FCP from the crude PSII and succeeded in preparation of a highly active PSII complex lacking major components of FCP but retaining the five extrinsic proteins. Various properties were compared between the crude and purified PSII, and the purified PSII was found to be much stable compared with the crude PSII.

2. Materials and methods

2.1. Culture of Chaetoceros gracilis

A marine centric diatom, *C. gracilis*, was grown in artificial seawater at 25 °C under continuous illumination at 30–35 μmol photons m^{-2} s^{-1} for 10 days, as described previously [4].

2.2. Isolation of the crude and purified PSII

The crude PSII from *C. gracilis* was prepared according to Nagao et al. [4] with slight modifications. In this study, the buffer containing 1 mM EDTA was used in preparation of thylakoid membranes and crude PSII to prevent cleavages of PSII components by endogenous proteases. The crude PSII was suspended in a medium containing 1 M betaine, 50 mM MES-NaOH (pH 6.5) and 1 mM EDTA (buffer A) at 1 mg Chl ml^{-1} and treated with 1% Triton X-100 for 20 min at 0 °C in the dark. After passing through a Millex-GS 0.22 μm filter (Millipore), the treated samples were applied to a DEAE-Toyopearl 650M column equilibrated with buffer A containing 0.03% Triton X-100. The column was washed with 2–3 bed volumes of the equilibrating buffer, and then a green fraction (PSII lacking major components of FCP) was first eluted at 180 mM NaCl, followed by a brownish-green fraction (PSII containing a small amount of FCP). A major FCP (brown fraction) was eluted at 1 M NaCl. The green fraction was collected and concentrated by centrifugation at 40,000×*g* for 20 min after addition of 10% polyethylene glycol 6000. The resulting precipitates (purified PSII complexes) were suspended in a medium containing 40 mM MES-NaOH (pH 6.5) and 0.4 M sucrose (buffer B), and stored at −196 °C. Thus, the purified PSII was isolated by a simple method, namely, one step of anion exchange chromatography within 2 h after treatment of the crude PSII with Triton X-100. The yield of the purified PSII was about 6% of the crude PSII on the basis of Chl *a*.

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were solubilized with 5% lithium lauryl sulfate and 75 mM dithiothreitol for 30 min at room temperature. The solubilized samples (3 μg Chl *a* for the crude PSII and 1 μg Chl *a* for the purified PSII) were applied to a gradient gel containing 16–22% acrylamide and 7.5 M urea according to Ikeuchi and Inoue [16]. Electrophoresis was carried out at a constant current of 8 mA for 15 h at room temperature. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 and photographed.

2.4. Assay of oxygen-evolving activity

Oxygen evolution was measured with a Clark-type oxygen electrode at 25 °C in buffer B containing 10 μg Chl *a* for crude PSII or 5 μg Chl *a* for purified PSII under saturating light. As electron acceptors, 0.4 mM phenyl-*p*-benzoquinone (PBQ), 0.5 mM 2,6-dichloro-*p*-benzoquinone (DCBQ) or 2 mM potassium ferricyanide (hereafter referred to as ferricyanide) was used.

2.5. Analysis of pigments and plastoquinone

The contents of pigments and plastoquinone (PQ) were determined by reversed-phase high-performance liquid chromatography with a Prodigy 5 (ODS 3100Å) 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 [17]. Chlorophyll concentrations were determined in 90% acetone using the equation of Jeffrey and Humphrey [18].

2.6. Absorption and fluorescence spectra

Absorption spectra were measured using a Cary 500 spectrophotometer at −193 °C. For low-temperature spectra, a cryostat (OptistatDN, Oxford Inst. Oxford, UK) was used in conjunction with a controller (Oxford ITC-601PT). Fluorescence spectra at −196 °C were measured with a Hitachi 850 spectrofluorometer (Hitachi, Japan) with a custom-made Dewar system as described previously [19]. Polyethylene glycol (average molecular weight 3350, final concentration 15% (w/v), Sigma-Aldrich, USA) was added to sample solutions to obtain homogeneous ice at −196 °C. The spectral sensitivity of the fluorometer was corrected by using a sub-standard lamp with a known radiation profile (Hitachi) as described previously [19].

2.7. Stability of the crude and purified PSII

The crude and purified PSII (1 mg Chl *a* ml^{-1}) in buffer B were incubated at 25 °C in the dark for various times, and then their oxygen-evolving activities, absorption spectra and polypeptide

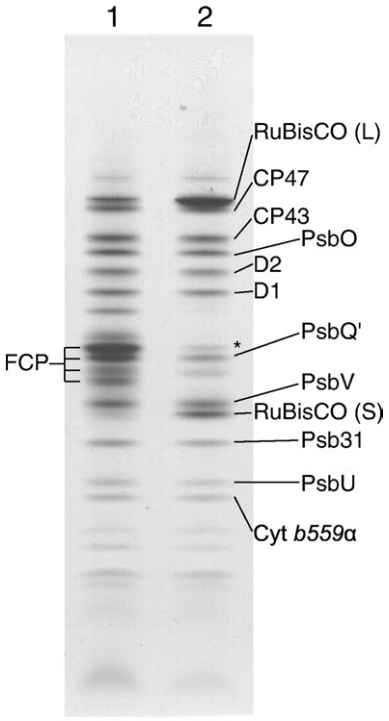

Fig. 1. Polypeptide compositions of the crude (lane 1) and purified (lane 2) PSII isolated from *Chaetoceros gracilis*. Each polypeptide was identified by immunoblotting analysis and N-terminal sequencing according to the methods described previously [4]. An asterisk in lane 2 indicates a FCP minor band.

Table 1
Effects of electron acceptors, DCMU and $CaCl_2$ on oxygen evolution of the crude and purified PSII isolated from *Chaetoceros gracilis*.

Oxygen evolution (μmol O_2 (mg Chl *a*)$^{-1}$ h^{-1})[a]			
	Acceptors	−ion	+5 mM $CaCl_2$
Crude PSII	PBQ	832 ± 145	891 ± 191
	+ DCMU	0	0
	DCBQ	604 ± 117	616 ± 103
	Ferricyanide	109 ± 23	142 ± 17
	PBQ	2135 ± 77	2170 ± 68
Purified PSII	+ DCMU	0	0
	DCBQ	1289 ± 63	1289 ± 63
	Ferricyanide	496 ± 74	1361 ± 132

[a] The values shown are averages from three separate measurements.

compositions were examined. Oxygen-evolving activity was measured with PBQ as the electron acceptor as described above, and plotted against incubation times. Absorption spectra were measured using a Beckman Coulter DU 800 spectrophotometer at room temperature, and the decrease at 440 nm (Chl bleaching) during the incubation was plotted against incubation times. Multicomponent deconvolutions of the inactivation and Chl bleaching curves were carried out using a fitting function with single or double exponential algorithm contained in IGOR Pro v. 5.05 (Wavemetrics, USA). The quality of fits was judged using a reduced χ^2 criterion and plots of the weighted residuals. Degradation of PSII subunits during the incubation was analyzed by SDS-PAGE described above. These measurements were carried out immediately after isolation of the crude PSII because the PSII was significantly labile as described later.

3. Results

3.1. Polypeptide compositions of the crude and purified PSII

Fig. 1 shows polypeptide compositions of the crude and purified PSII. Each polypeptide was identified by immunoblotting analysis with antibodies raised against CP47, CP43, PsbO, D2, D1, FCP, PsbQ', PsbV, Psb31, PsbU and α subunit of cytochrome *b*559, and by N-terminal sequencing for the large (L) and small (S) subunits of ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO), according to the methods described previously [4]. Most of the major bands of FCP in the crude PSII (lane 1) were removed in the purified PSII (lane 2), but the five extrinsic proteins of PsbO, PsbQ', PsbV, Psb31 and PsbU were retained in the purified PSII (lane 2). Thus, the purified PSII consisted of PSII core subunits CP47, CP43, D2, D1, α subunit of cytochrome *b*559 and several small subunits in addition to the five extrinsic proteins (lane 2). However, the purified PSII contained a large amount of the large (L) and small (S) subunits of RuBisCO (lane 2). We attempted to separate RuBisCO from PSII. When the Triton-treated crude PSII was applied to DEAE-Toyopearl 650M column and then eluted with 0–1 M NaCl gradient, RuBisCO subunits were eluted at lower concentrations of NaCl and separated from PSII complexes. However, a small amount of the extrinsic proteins were eluted together with RuBisCO subunits, and the PSII separated from RuBisCO showed a lower oxygen-evolving activity. Thus, we used PSII containing RuBisCO in this study.

3.2. Oxygen-evolving activity of the crude and purified PSII

Table 1 shows oxygen-evolving activities of the crude and purified PSII in the absence and presence of 5 mM $CaCl_2$ using various electron acceptors. The activity of the purified PSII in the absence of $CaCl_2$ was 2135 μmol O_2 (mg Chl *a*)$^{-1}$ h^{-1} with PBQ as the electron acceptor, which was more than 2.5-fold higher than the activity of the crude PSII. The activity was virtually not stimulated by the addition of 5 mM $CaCl_2$ and completely inhibited by 10 μM DCMU, indicating that the purified PSII has preserved their intactness at both the acceptor and donor sides after isolation. The activity of the purified PSII in the absence of $CaCl_2$ decreased to 1289 μmol O_2 (mg Chl *a*)$^{-1}$ h^{-1} with DCBQ as the acceptor, and further to 496 μmol O_2 (mg Chl *a*)$^{-1}$ h^{-1} with ferricyanide as the acceptor. These properties of oxygen-evolving activity of the purified PSII were similar to those of the crude PSII. However, when ferricyanide was used as the electron acceptor, the activity of the purified PSII, especially in the presence of $CaCl_2$, was significantly higher than that of the crude PSII. This may be related to a partial release of PQ in the purified PSII during its isolation (see Table 2). It seems likely that release of PQ from Q_B-binding site leads to electron transfer from Q_A to ferricyanide, in which Ca^{2+} ions increase the affinity of ferricyanide to the Q_A site by interaction of Ca^{2+} ions with the negative ions of ferricyanide and around the Q_A site, as described previously [9]. Haag et al. [20] also showed that the action of ferricyanide versus PBQ or DCBQ on oxygen evolution was quite different in PSII core preparation from plants, and this difference might be due to the properties of the Q_B site which modified the access of the negatively charged ferricyanide.

3.3. Pigments and plastoquinone composition of the crude and purified PSII

Table 2 shows the amounts of pigments and PQ in the crude and purified PSII. On the basis of two molecules of pheophytin *a*, 86 molecules of Chl *a*, 32 molecules of Chl *c*, 64 molecules of fucoxanthin, 5 molecules of diadinoxanthin, 7 molecules of β-carotene and 2 molecules of PQ were contained in the crude PSII. These values are different from those reported in our previous paper [4]. This was found to be due to an underestimation of pheophytin *a* (about 0.45-fold) in our previous paper, leading to the significantly larger values of the pigments in the previous report. We would like to correct them in this paper. The values corrected with valid amount of pheophytin *a* in the previous paper were 103.05 Chl *a*, 38.7 Chl *c*, 72.9 fucoxanthin, 5.94 diadinoxanthin, and 7.00 β-carotene, respectively, which are comparable with those in the present study. On the other hand, the purified PSII contained 42 molecules of Chl *a*, 2 molecules of Chl *c*, 6 molecules of fucoxanthin, 2 molecules of diadinoxanthin, 7 molecules of β-carotene and 1.4 molecules of PQ on the basis of two molecules of pheophytin *a*. This indicates that most of Chl *c* and fucoxanthin in the crude PSII were removed in the purified PSII together with major FCP components, while 48% of Chl *a*, 40% of diadinoxanthin and all of β-carotene in the crude PSII were remained in the purified PSII. It is noted that 2 molecules of diadinoxanthin, a key substance in the xanthophyll-cycle in diatoms [17], were still associated with the purified PSII. These diadinoxanthin seem to bind to a minor

Table 2
Amounts of pigments and plastoquinone associated with the crude and purified PSII isolated from *Chaetoceros gracilis*.

Pigments and plastoquinone (molar ratio to 2 pheophytin *a*)[a]						
	Chl *a*	Chl *c*	Fucoxanthin	Diadinoxanthin	β-carotene	PQ
Crude PSII	85.7 ± 1.27	31.9 ± 0.49	64.4 ± 1.19	5.2 ± 0.08	6.6 ± 0.15	2.11 ± 0.04
Purified PSII	41.5 ± 0.36	2.2 ± 0.02	5.7 ± 0.02	2.1 ± 0.02	7.2 ± 0.08	1.42 ± 0.02

[a] The values shown are averages from three separate measurements.

component of FCP associated with the purified PSII, because a minor FCP band was observed in SDS-PAGE of the purified PSII (asterisk in lane 2 of Fig. 1), and a small amount of Chl *c* and fucoxanthin were contained in the purified PSII (Table 2).

3.4. Absorption and fluorescence spectra of the crude and purified PSII

Absorption spectra of the crude (dotted line) and purified (solid line) PSII at −193 °C are shown in Fig. 2. The crude PSII showed absorption maxima at 669.0 and 437.5 nm due to Chl *a* and at 634.0 and 457.0 nm contributed by Chl *c*, while the purified PSII showed absorption maxima at 669.5 and 435.5 nm due to Chl *a* but no Chl *c* bands, indicating that most of Chl *c* has been removed together with FCP. Furthermore, the absorption between 450 and 560 nm due to fucoxanthin was remarkably decreased in the purified PSII. Absorption maxima at 493.0 and 460.0 nm due to β-carotene clearly appeared in the purified PSII by removal of fucoxanthin. Prominent shoulders around 680 nm were also detected in the purified PSII, and those were resolved to 677.0 and 683.0 nm by the second derivative of the spectrum. In addition, an apparent maximum of the Soret band at 435.5 nm was resolved to 436.5 nm by the second derivative spectrum. These patterns were similar to the purified PSII from *Synechocystis* sp. PCC 6803 [19]. These components, however, were not clearly separated in the crude PSII due to overlapping absorptions by Chl *a* from FCP.

Fig. 3 shows fluorescence emission spectra at −196 °C of the crude (A) and purified (B) PSII upon excitation at 435 nm, 460 nm or 495 nm. The fluorescence spectra of the crude PSII displayed a maximum at 686.2 nm and a shoulder around 695 nm, irrespective of the excitation wavelengths. When the crude PSII was excited at 460 nm, a fluorescence band derived from Chl *c* was observed at 638.2 nm, suggesting a somewhat lower energy transfer from Chl *c* to Chl *a* in the crude PSII. On the other hand, the fluorescence spectra of the purified PSII displayed maxima at 687.2 and 694.4 nm, irrespective of the excitation wavelengths. The two typical fluorescence bands are consistent with those in the purified higher plant and cyanobacterial PSII [21,22]. A vibrational sub-band around 755 nm was also observed in both the crude and purified PSII, which is similar to that observed in the purified higher plant and cyanobacterial PSII [21,22].

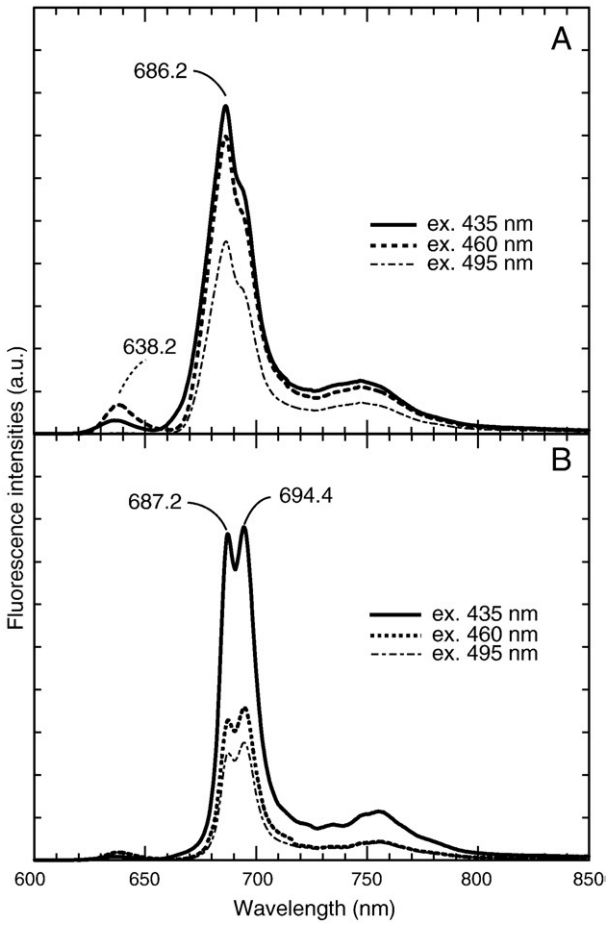

Fig. 3. Fluorescence spectra at −196 °C of the crude (A) and purified (B) PSII isolated from *Chaetoceros gracilis*. Excitation wavelengths were selected to excite the Soret band of Chl *a* (435 nm), Chl *c* (460 nm), and carotenoids (495 nm).

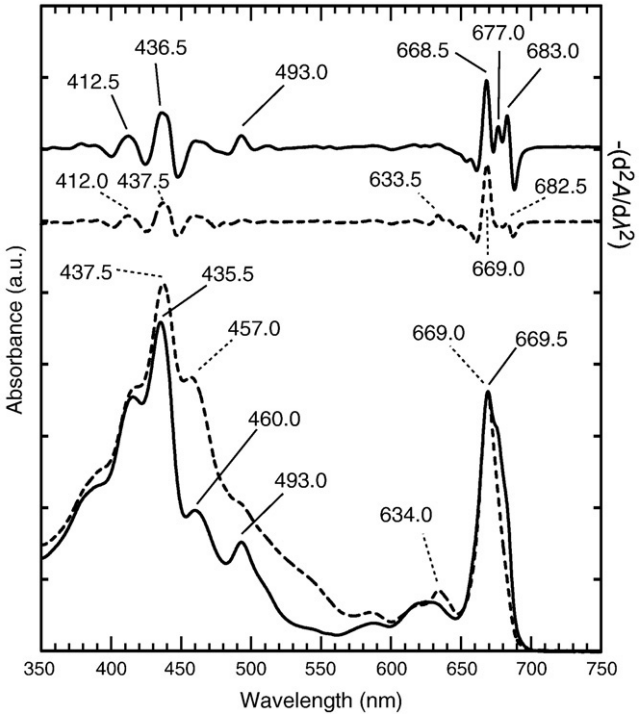

Fig. 2. Absorption spectra (below) and their second derivative spectra (above) at −193 °C of the crude (dotted line) and purified (solid line) PSII isolated from *Chaetoceros gracilis*.

3.5. Stability of the crude and purified PSII

3.5.1. Inactivation of oxygen evolution

Surprisingly, the crude PSII prepared from *C. gracilis* was significantly labile. When the crude PSII was incubated in buffer B at 25 °C in the dark, the oxygen-evolving activity decreased to 80%, 68%, 60%, 55%, 45%, 9% and 0% of the original activity after incubation for 5 min, 10 min, 20 min, 30 min, 60 min, 3 h and 6 h, respectively (dotted line in Fig. 4). Deconvolution analysis of the measured inactivation curve showed that the curve consisted of two components with a fast phase (4.2 min, 25.4%) and slow phase (98.5 min, 74.6%), respectively (Table 3). In contrast, the activity of the purified PSII scarcely decreased within 60 min and retained about 90% and 80% of the original activity even after incubation for 3 h and 6 h, respectively (solid line in Fig. 4). The inactivation curve of the purified PSII consisted of only a slow phase (1550 min) (Table 3), which was much slower than the slow phase of the crude PSII. These suggest that the biphasic kinetics in the inactivation of oxygen evolution is a feature of the crude PSII itself but not due to possible heterogeneity of the sample. In any cases, these results indicate that the purified PSII is much stable compared with the crude PSII, suggesting that the oxygen-evolving activity is significantly stabilized probably by removal of an inactivating factor(s) during the purification procedures.

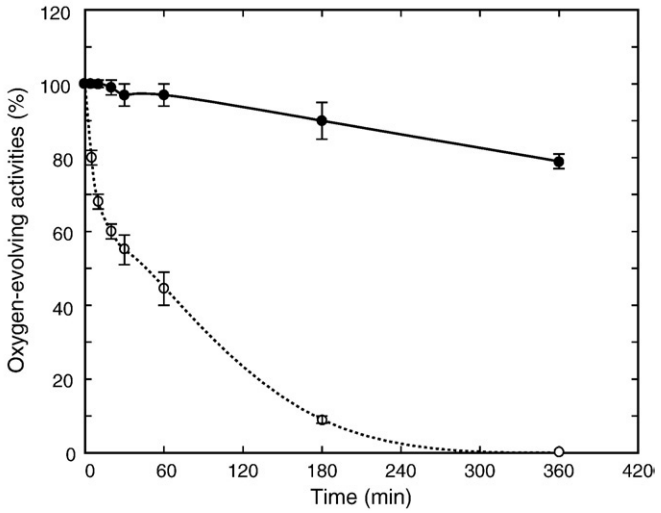

Fig. 4. Stability of oxygen-evolving activity in the crude (dotted line) and purified (solid line) PSII. After incubation of the crude and purified PSII in a medium of 40 mM MES-NaOH (pH 6.5) and 0.4 M sucrose (buffer B) at 25 °C in the dark for various times, the oxygen-evolving activity was measured at 25 °C in buffer B with 0.4 mM phenyl-*p*-benzoquinone as the electron acceptor.

3.5.2. Chl bleaching

A remarkable Chl bleaching was found in the crude PSII. When the crude PSII was incubated at 25 °C in the dark, the absorbance at 672 nm and 440 nm due to Chl *a* and at 638 nm and 458 nm due to Chl *c* decreased with the incubation times. Fig. 5A shows the difference absorption spectra of after incubation minus before incubation of the crude PSII at room temperature. The difference spectra showed significant decreases in the typical absorption peaks of Chl *a* and Chl *c*, indicating specific degradation of Chl during the dark incubation. The negative peak at 532 nm appeared after 6 h (solid line) seems to be due to FCP bleaching. In contrast, the Chl bleaching in the purified PSII was much slower than that in the crude PSII. Fig. 5B shows the time courses of the Chl bleaching in which the absorption decrease at 440 nm was plotted against incubation times. Deconvolution analysis of the measured curve showed that the Chl bleaching of the crude PSII consisted of two components with a fast phase (19.9 min, 9.2%) and slow phase (2750 min, 90.8%), respectively, while that of purified PSII consisted of only one component corresponding to the slow phase (3488 min) (Table 3). These suggest that a factor(s) responsible for the fast Chl bleaching in the crude PSII has been removed in the purified PSII.

3.5.3. Degradation of PSII subunits

Most of subunits in the crude PSII were found to be degraded during incubation at 25 °C in the dark. As shown in Fig. 6A, the

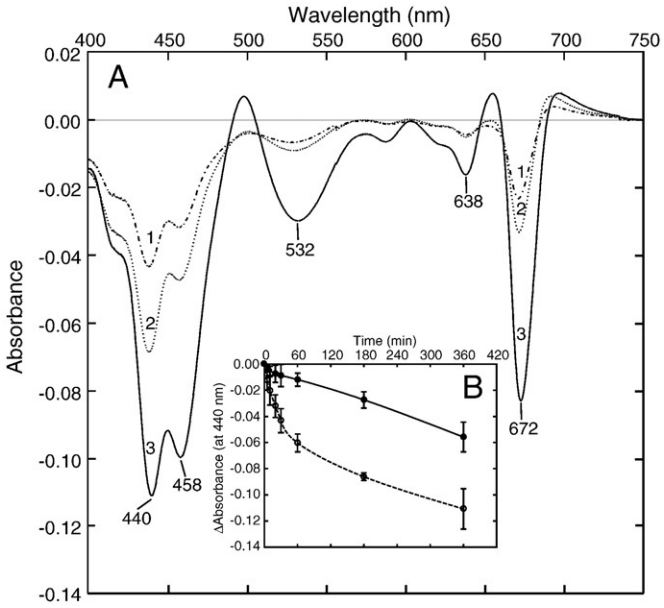

Fig. 5. Difference absorption spectra at room temperature of the crude PSII (A) and time courses of Chl bleaching at 440 nm (B). After incubation of the crude PSII at 25 °C in the dark for 30 min (1), 1 h (2) or 6 h (3), the difference absorption spectra of after incubation minus before incubation were measured at room temperature (A). Decreases of absorption at 440 nm of the crude (dotted line) and purified (solid line) PSII after the dark incubation for various times were plotted against incubation times (B).

bands of CP47, CP43, PsbO, D2, D1, an unknown band migrated below D1, four FCP subunits, PsbQ', PsbV, Psb31 and PsbU gradually disappeared with the incubation times, and these bands were

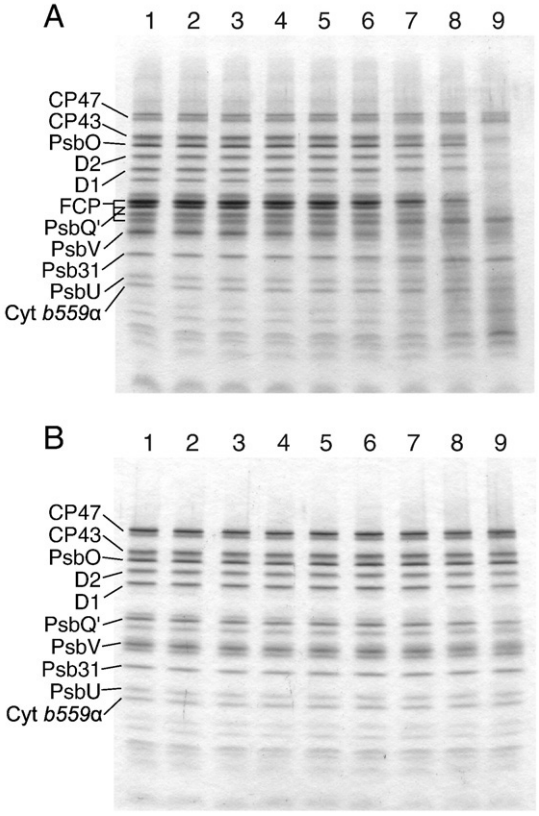

Fig. 6. Degradation of PSII subunits in the crude (A) and purified (B) PSII during incubation at 25 °C in the dark for various times. The crude and purified PSII were incubated at 25 °C in the dark for 0 min (lane 1), 5 min (lane 2), 10 min (lane 3), 20 min (lane 4), 30 min (lane 5), 1 h (lane 6), 3 h (lane 7), 6 h (lane 8) or 18 h (lane 9), and then analyzed by SDS-PAGE.

Table 3
Rate constants and relative amplitudes of the inactivation of oxygen evolution and Chl bleaching processes.

Inactivation of oxygen evolution	Crude PSII		Purified PSII
	Fast phase	Slow phase	Slow phase
τ (min)	4.2	98.5	1550
Amplitude (%)	25.4	74.6	100

Chl bleaching	Crude PSII		Purified PSII
	Fast phase	Slow phase	Slow phase
τ (min)	19.9	2750	3488
Amplitude (%)	9.2	90.8	100

almost completely disappeared after 18 h (lane 9). Among these bands, it should be noted that the CP47 and Psb31 bands were shifted to bands migrated below the original bands by cleavage of these proteins. In contrast, these degradations were scarcely detected in the purified PSII, though the band intensities of D1, PsbQ' and PsbV were slightly decreased (Fig. 6B). These indicate that a highly active protease(s) is contained in the crude PSII and the protease(s) has been removed in the purified PSII during the purification process.

4. Discussion

In this study, a highly active PSII complex lacking major components of FCP but retaining the five extrinsic proteins was purified by a simple method involving one step of anion exchange chromatography from the crude PSII of a marine centric diatom, *C. gracilis*. The purified PSII showed a high oxygen-evolving activity of 2135 μmol O_2 (mg Chl *a*)$^{-1}$ h^{-1} in the absence of $CaCl_2$ (Table 1), and contained PSII core subunits of CP47, CP43, D2, D1, α subunit of cytochrome *b*559 and the five extrinsic proteins of PsbO, PsbQ', PsbV, Psb31 and PsbU (Fig. 1). Absorption and fluorescence spectra of the purified PSII were shown to be similar to those of highly purified PSII from cyanobacteria (Figs. 2 and 3). Thus, the diatom PSII isolated in this study is a highly purified PSII preparation comparable to the PSIIs purified from cyanobacteria, red algae and green algae [6–11,19,23] in terms of oxygen-evolving activity, absorption and fluorescence spectra, and polypeptide composition except for the presence of RuBisCO. The antenna size of the purified PSII was estimated to be 42 Chl *a* (Table 2), which is similar to the antenna size of 41 Chl *a* in purified cyanobacterial PSII from *Synechocystis* sp. PCC 6803 [11] but is larger than that of PSII core complex from thermophilic cyanobacteria (35–36 Chl *a*) [24,25]. This may be due to association of minor components of FCP (Fig. 1).

One of the interesting characteristics of the diatom PSII is that the crude PSII is significantly labile. The oxygen-evolving activity in the crude PSII was significantly inactivated during the dark incubation at 25 °C (Fig. 4). So far, we have prepared PSII membrane fragments and core complexes from cyanobacteria, red algae, Euglena, green algae and higher plants but such significant inactivation observed in the crude PSII was not observed in any PSII preparations (data not shown). In addition to the inactivation, a remarkable Chl bleaching was observed in the crude PSII during the dark incubation (Fig. 5). Chl bleaching in the dark is known to occur by enzymatic degradation of Chl [26]. Thus, it seems likely that enzymes responsible for the fast Chl degradation (Fig. 5B) are contained in the crude PSII. In fact, the highly active chlorophyllase activity has been reported to be present in diatoms among a number of algae tested [27–29]. Localization and function of Chl degradation enzymes containing chlorophyllase in the crude PSII will be interesting subjects for further characterization of diatom PSII.

Furthermore, we found significant degradation of PSII subunits in the crude PSII during the dark incubation (Fig. 6). The degradation occurred randomly in most of PSII subunits. Such degradations were not observed in cyanobacterial, red algal, green algal and spinach PSIIs even after dark incubation at 25 °C for 18 h (data not shown). On the other hand, when spinach PSII was incubated at 25 °C in the dark for 18 h together with the crude diatom PSII, most of the subunits in the spinach PSII as well as the diatom PSII were randomly degraded (data not shown). These clearly indicate that highly active proteases are contained in the crude PSII. Isolation and characterization of these highly active proteases in the crude PSII are now in progress in our laboratory.

Significant inactivation of oxygen evolution, Chl bleaching and degradation of PSII subunits are interesting characteristics specifically observed in the crude diatom PSII. Although the relationships among these phenomena is ambiguous at present, analyses of their time courses suggest that the inactivation of oxygen evolution first occurs, followed by Chl bleaching and then degradation of PSII subunits. We will study these phenomena in relation to the damage and repair cycle of the diatom PSII.

In contrast, the purified PSII obtained in this study is much stable compared with the crude PSII. The inactivation of oxygen evolution, Chl bleaching and degradation of PSII subunits observed in the crude PSII were largely suppressed in the purified PSII (Figs. 4, 5 and 6). This suggests that an inactivating factor(s) of oxygen evolution, Chl degradation enzymes responsible for Chl bleaching and proteases participated in degradation of PSII subunits have been removed together with FCP during the purification processes. Thus, we succeeded for the first time in isolation of a stable PSII preparation from diatom cells. The stable PSII can be effectively used for various biochemical studies, e.g. binding and functional analysis of the five extrinsic proteins in diatom PSII by release-reconstitution experiments which is now in progress.

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