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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

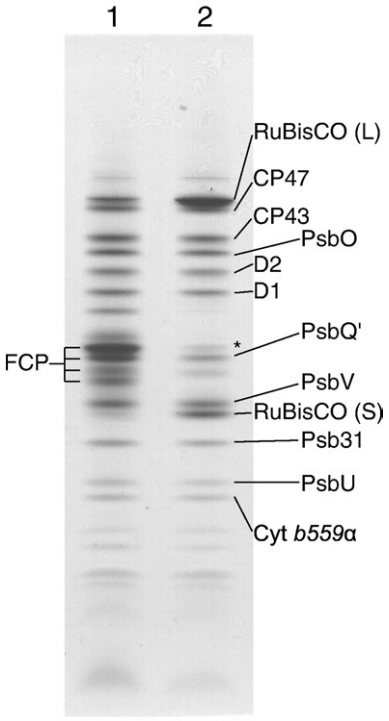

Fig. 1. Polypeptide compositions of the crude (lane 1) and purified (lane 2) PSII isolated from *Chaetoceros gracilis*. Each polypeptide was identified by immunoblotting analysis and N-terminal sequencing according to the methods described previously [4]. An asterisk in lane 2 indicates a FCP minor band.