

Isolation and characterization of photosystem II from the filamentous sporophyte of *Porphyra yezoensis*

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Thylakoid membranes were isolated and purified from diploid filamentous sporophytes of *Porphyra yezoensis* Ueda using sucrose density gradient ultracentrifugation (SDGUC). After thylakoid membranes were solubilized with SDS, the photosystem II (PSII) particles with high 2, 6-dichloroindophenol (DCIP) photoreduction activity were isolated by SDGUC. The absorption and fluorescence spectra, DCIP photoreduction activity and oxygen evolution activity of the thylakoid membranes and PSII particles were determined. The polypeptide composition of purified PSII particles was distinguished by SDS-PAGE. Results showed that PSII particles of sporophytes differed from the gametophytes in spectral properties and polypeptide composition. Apart from 55 kDa D1-D2 heterodimer, CP47, CP43, 33 kDa protein, D1, D2, cyt b559 and 12 kDa protein were identified from PSII particles from sporophytes; a new 102 kDa protein was also detected. However, cyt *c*-550, 20 kDa, 14 kDa and 16 kDa proteins found in PSII particles from gametophytes were not detected in the sporophytes.

Keywords: *Porphyra yezoensis*, Photosystem II, Filamentous sporophytes, Thylakoid membranes, Polypeptide composition, Sucrose density gradient ultracentrifugation

Photosynthesis is the vital process by which plants, algae and photosynthetic bacteria utilize the solar energy to fix carbon dioxide (CO₂), produce organic compounds, oxidize water, and release oxygen. Isolation and characterization of pigment-protein complexes, including photosystem I and II (PSI and PSII), the key players in photosynthesis, is one of the important areas of interest in photosynthetic investigation. PSII is an important photosynthetic complex responsible for splitting water, transporting free electron and releasing proton to thylakoid lumen as well as evolving oxygen, and hence finds use as a phylogenetic research target¹⁻³.

In recent years, significant progress has been made on structure and function of PSII of higher plants and cyanobacteria⁴. Two-dimensional structure of spinach PSII at 8 Å resolution⁵, and crystal structures of PSII

from *Synechococcus elongatus*⁶, oxygen-evolving complex PSII at 3.7 Å resolution from *Thermosynechococcus vulcanus*⁷, cyanobacterial PSII complex at 3.5 Å resolution from *T. elongatus*⁸, as well as spinach major light-harvesting complex II at 2.72 Å resolution have been reported recently⁹. However, studies on algal pigment-protein complexes are still lacking. In fact, algae as transitional link from photosynthetic bacteria to higher plants are ideal for investigation for comparative photosynthesis and evolution of photosynthetic organisms^{10,11}.

In red algae, studies have been focused mainly on unicellular, acidophilic and thermophilic freshwater algae, such as *Porphyridium cruentum* and *Cyanidium caldarium*¹²⁻¹⁷. But, PSII of multicellular red algae, such as *Porphyra* and *Gracilaria* has not received much attention. *Porphyra yezoensis*, a common seaweed, distributed widely in East-Asia¹⁸ is one of the largest aquaculture industries in China and Japan. It is easy to grow in culture and can produce large amounts of biomass in relatively short time¹⁹. It has unique metagenesis and interesting life history with two life phases (unlike most of the red algae which have three distinct phases) — haploid blade gametophyte, and diploid filamentous sporophyte that can

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Abbreviations: Chl, chlorophyll; cyt, cytochrome; DCIP, 2, 6-dichloroindophenol; DIG, digitonin; DPC, 1, 5-diphenylcarbohydrazide; OG, octylglucoside; Pheo, pheophytin; PSII, photosystem II; SDGUC, sucrose density gradient ultracentrifugation; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

live independently. Moreover, unlike higher plants and other algae, its gametophytes are bigger than sporophyte.

Earlier, we have isolated and characterized PSII particles from *P. yezoensis* gametophytes and found their spectral characteristics and composition different from that of other plants^{20,21}. In the present study, PSII particles with high photoreduction activity have been isolated from sporophytes of *P. yezoensis*. Their peptide composition and different photosynthetic properties have been investigated and compared with the gametophytes.

Materials and Methods

Material and culture condition

The healthy, large and mature gametophytes without differentiation collected in mid-April were cleaned and dried in shade. After 3–5 days, the desiccated gametophytes were cultivated in bacteria-free medium deposited beforehand for a week in dark. Medium was stirred continuously and density of released carpospores was observed under microscope. When carpospores density was 10,000/ml, the medium was filtered and the filtrate was inoculated in conical flasks and kept at 17°C with air exchange, and 1800 lx illumination for 12 h light/dark cycle. Culture medium was changed every week. The villiform filamentous sporophytes (conchocelis) observed on the bottom of conical flasks after a month were cut into several segments and cultivated again in bacteria-free medium to enlarge biomass. After 3–4 months, the sporophytes were harvested, washed with filtered seawater thrice, dipped in distilled water for 10 min and dried with filter paper.

Preparation of crude thylakoid membranes

Filamentous sporophytes (30 g) were fragmented in a triturator containing 75 ml cold extract buffer (50 mM Tris, 5 mM EDTA, 1 mM MnCl₂, 1 mM MgCl₂, 2 mM NaNO₃, 100 mM sucrose, 0.5 mM K₂HPO₄, pH 7.8) for 30 min at 4°C, and grinded further at 0°C in dark for 60 min and filtered with 8 layers of cold gauze. The filtrate was centrifuged at 2,000 × *g* for 10 min at 4°C (Eppendorf centrifuge 5804R) and the supernatant was centrifuged again at 10,000 × *g* for 30 min. The resultant pellet, crude thylakoid membranes was suspended in cold extract buffer without sucrose in dark.

Purification of thylakoid membranes and isolation of PSII particles with SDGUC

The suspended crude thylakoid membranes were placed on to the first SDGUC consisting of 60%,

50%, 40%, 30%, 20% (w/v) sucrose in proportion of 1:1:1:1:1. The gradients were centrifuged at 14,000 × *g* (Beckman L8-80, Ti-45 rotor) for 4 h at 4°C. The bands with different colors were dialyzed against cold extract buffer without sucrose respectively at 4°C in dark to remove sucrose.

For isolation of PSII particles, the dialyzed bands were treated with different concentrations of Triton X-100, DIG, OG and SDS, respectively in dark at 4°C for 30 min with continuous stirring. The concentration of Chl *a* was determined as described previously²². Later, the bands were placed on to the SDGUC consisting of 60%, 50%, 40%, 30%, 20%, 15%, 10% (w/v) sucrose density in proportion of 1:1:1:1:1:1:1, and centrifuged at 140,000 × *g* for 20 h at 4°C. The fractions with different colors were dialyzed against cold extract buffer without sucrose to remove sucrose.

Determination of DCIP photoreduction activity and absorption and fluorescence spectra of PSII particles/thylakoid membranes

To identify PSII particles, their DCIP photoreduction activity was determined. The rates of DCIP photoreduction of the bands obtained by the second SDGUC were measured according to the methods described previously^{21,23,24}. Absorption spectra of PSII particles and thylakoid membranes were recorded using a Beckman DU 650 spectrophotometer. Fluorescence spectra were detected with a Hitachi F-4500 fluorescence spectrophotometer.

Oxygen evolution activity

Oxygen evolution activity of samples was measured according to previously described methods^{24,25} at 20°C under irradiation with a Clark-type oxygen electrode (Hansatech) in the medium containing 0.4 M sucrose, 30 mM CaCl₂, 600 μM phenyl-*p*-benzoquinone, 2 mM ferricyanide kalium and 50 mM Mes-NaOH (pH 6.0), with saturated 'white light'.

SDS-PAGE

SDS-PAGE was used to examine polypeptides composition of PSII particles, according to Laemmli²⁶. Samples were first precipitated with 9 vols of cold acetone, mixed with loading buffer (0.25 M Tris, 5% glycerol, 1% SDS, 0.025% β-mercaptoethanol, and applied to gel with prior heating in boiling water for 5 min. Separating and stacking gels were 12% (pH 8.8), and 5% (pH 6.8), respectively. After electrophoresis, the gel was

stained with Coomassie brilliant blue (CBB G₂₅₀) (4.75% ethanol, 8.5% H₃PO₄, 0.0001% CBB G₂₅₀) and de-stained with water for 6 h. For demonstration of extrinsic proteins of active PSII particles from sporophyte of *P. yezoensis*, the PSII particles were washed with alkaline Tris and 1 M CaCl₂^{20,27}.

Results

Isolation and purification of thylakoid membranes

After the first SDGUC of thylakoid membranes, four bands with different colors appeared in centrifuge tubes (Fig. 1a). Results from absorption and fluorescence emission spectra suggested A₁, A₂ and A₃ were purified thylakoid membranes which had similar spectra properties while A₄ was phycoerythrin.

Isolation of PSII particles from purified thylakoid membranes

Among all the conditions tested using detergents DIG, OG, Triton X-100 and SDS to solubilize purified thylakoid membranes, SDS and Chl *a* in the ratio of 25:1 (w/w) was the most effective, yielding the clearest bands. Different results were obtained from A₁, A₂, and A₃ with 25 (SDS): 1 (Chl *a*) (w/w), though they all proved to be purified thylakoid membranes. When purified thylakoid membranes were solubilized with 25 (SDS): 1 (Chl *a*) and centrifuged by the second time SDGUC, six clear bands (marked A₁-a to A₁-f) were obtained from A₁ (Fig. 1b), while three and four blurred bands were obtained from A₃ and A₂, respectively (data not shown). A₁, A₂ and A₃ when treated with DIG, OG, Triton X-100 and 50 or 100 (SDS): 1 (Chl *a*) (w/w) did not yield better results.

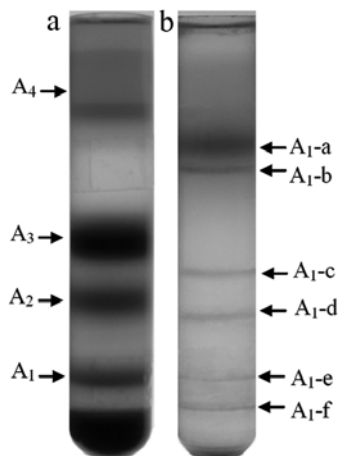


Fig. 1—(a): Purification of thylakoids membranes by sucrose density gradient ultracentrifugation. A₁, A₂ and A₃ were purified thylakoid membranes, and A₄ was phycoerythrin; (b): Isolation of PSII particles from purified thylakoids membrane A₁. Before ultracentrifugation, the samples were treated with SDS at the ratio 25 (SDS): 1 (Chl *a*)

Absorption and fluorescence spectra

Fig. 2 shows the absorption spectra of purified thylakoid membranes A₁ (curve 1) and PSII particles (A₁-f, curve 2). Two prominent peaks at 418 nm and 673 nm, a low peak at 622 nm and two shoulders at 437 nm and 484 nm were observed in absorption spectra of A₁. No distinct peak of phycoerythrin at 498, 545 and 565 nm indicated phycoerythrobilin was eliminated thoroughly in the samples. As for PSII particles (A₁-f), the prominent peaks were found at 419 nm and 672 nm. Besides, there were two low peaks at 506 nm and 618 nm and two shoulders at 435 nm and 539 nm.

Fig. 3 shows the fluorescence emission spectra of purified thylakoid membranes A₁ (curve 1) and PSII particles (A₁-f, curve 2) at room temperature. The

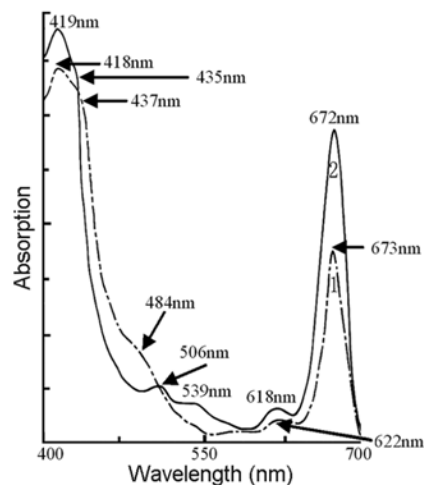


Fig. 2—Absorption spectra of purified thylakoid membranes A₁ (curve 1), and purified PSII particles (curve 2) at room temperature

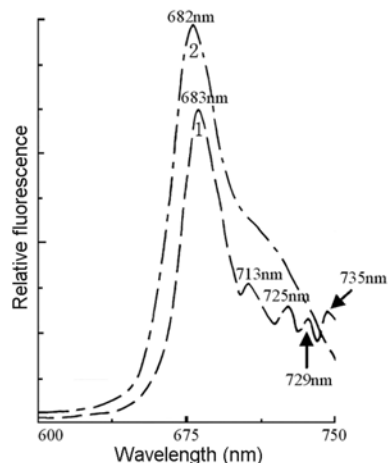


Fig. 3—Fluorescence emission spectra of A₁ (curve 1) and A₁-f (PSII particles, curve 2) at room temperature (Ex = 436 nm)

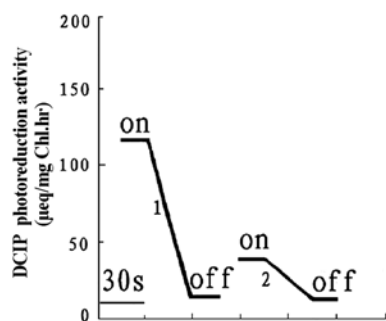


Fig. 4—DCIP photoreduction assay of PSII particles (A_1 -f). [Curve 1, with DPC as electron donor; curve 2, with H_2O as electron donor (CK)]

Table 1—Oxygen evolution activity of the samples

Samples	Oxygen evolution rate ($\mu\text{mol O}_2/\text{mg Chl h}$)
A_1	158.5
A_2	66.6
A_3	43.5
A_1 -a and A_1 -b	Nil
A_1 -c	05.8
A_1 -d	11.2
A_1 -e	18.2
A_1 -f (PSII particles)	233.5

purified thylakoid membranes A_1 exhibited a prominent emission peak at 683 nm, and four small peaks at 713, 725, 729 and 735 nm. As for PSII particles (curve 2), there was a prominent fluorescence emission peak at 682 nm near typical PSII emission peak ($E_x = 436 \text{ nm}$)²⁸.

Photochemical activity of PSII

Result of photoreduction rate of DCIP indicated that only A_1 -f was PSII particles, while no photochemical activity was detected in the bands from A_2 , A_1 -a and A_1 -b, while A_1 -c and A_1 -d had much lower activity compared to A_1 -f. Photochemical activity of PSII with DPC as artificial electron donor was 4-times higher than that of with H_2O as donor (Fig. 4).

Oxygen evolution activity

Results of oxygen evolution rate of the samples (Table 1) showed that PSII particles (A_1 -f) had the highest oxygen evolution activity ($233.5 \mu\text{mol O}_2/\text{mg Chl h}$), compared to the purified thylakoid membranes (A_1) ($158.5 \mu\text{mol O}_2/\text{mg Chl h}$). A_1 -a and A_1 -b exhibited no oxygen evolution activity, while A_1 -c had the least oxygen evolution activity ($5.8 \mu\text{mol O}_2/\text{mg Chl h}$).

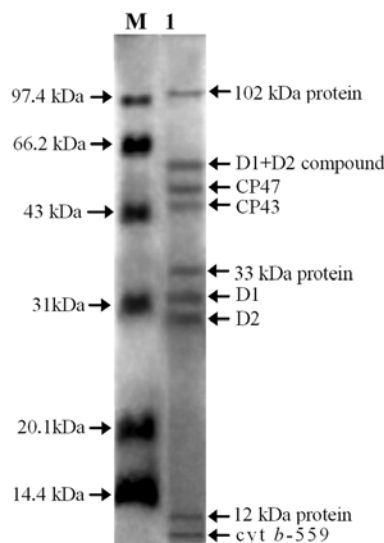


Fig. 5—Polypeptide composition of A_1 -f (PSII) [M, low molecular marker; Lane 1, A_1 -f (PSII)]. The bands were stained with CBB G_{250}]

Composition of PSII particles

SDS-PAGE of PSII particles showed 9 protein bands (Fig. 5) identified as 102 kDa protein (reported for the first time), 55 kDa D1 and D2 heterodimer, CP47, CP43, 33 kDa protein, D1, D2, 12 kDa protein and cyt *b*-559. However, cyt *c*-550, 20 kDa, 14 kDa and 16 kDa proteins found in PSII particles from gametophytes were not detected in the sporophytes.

Discussion

Among the photosynthetic organisms, red algae are one of the most primitive eukaryotic algae, phylogenetically closer to the prokaryotic cyanobacteria. Both use phycobilisomes as light-harvesting systems. Moreover, like cyanobacterium, thylakoids of red algae do not stack, but lie equidistantly and singly in chloroplasts²⁰. Both the origin of red algae and their relationship to other eukaryotic organisms have been disputed for over a century²⁹. Comparative sequence data from a growing number of nuclear genes suggest that red algae have diverged just before the common ancestor of plants, animals and fungi³⁰⁻³². Recent evidences suggest Rhodophytes either sister to green plants^{33,34} or the nearest relatives of a group of eukaryotes that includes all multicellular organisms with complex patterns of ontogenetic and tissue-specific development³⁵.

In terms of total polypeptide composition, red algae thylakoid polypeptides were found to be more similar

to higher plants than those of cyanobacteria³⁶. Moreover, a Chl *a*-binding light-harvesting protein associated with PSI was obtained from a unicellular freshwater red alga *P. cruentum*, indicating red algal PSI was more similar to higher plants PSI than cyanobacteria PSI^{12,13}. The PSII of *P. cruentum* is reported to have intrinsic subunits CP43, CP47, D1 and D2, and retained the extrinsic subunits of its oxygen-evolving complex, 33 kDa, cyt C550 and 12 kDa³⁷. However, PSII from an acidophilic and thermophilic red alga *C. caldarium* was phylogenetically closer to PSII of cyanobacteria than PSII of higher plants. PSII of both cyanobacteria and *C. caldarium* shared extrinsic cytochrome *c*-550 and 12 kDa protein¹⁴⁻¹⁶. The above-mentioned studies reveal phylogenetical importance of red algae, pertaining to evolution of photosystem, in particular. Therefore, understanding structure and components of photosystems of red algae has phylogenetic significance in evolutionary biology.

In the present study, five different properties were found between sporophytes and gametophytes. (i) After the first SDGC, four bands with different colors appeared from crude thylakoid membranes of sporophyte (Fig. 1a), of which three were the purified thylakoid membranes, while there were only three bands with different colors from crude thylakoid membranes of gametophyte, of which only two were purified thylakoid membranes²⁰.

(ii) Although absorption spectra of purified thylakoid membranes and PSII particles from sporophyte and gametophyte were almost similar, differences were found in their fluorescence emission spectra. Besides, the prominent fluorescence emission peak at 683 nm which shifted 2 nm to blue wave than the purified thylakoid membranes of gametophytes, there were other four lower peaks at 713, 725, 729 and 735 nm in that of sporophyte. Compared with fluorescence emission spectra of gametophytes, PSII particles of sporophytes had a prominent emission peak at 682 nm, shifting 3 nm to blue wave; in addition, there was another 723 nm shoulder²⁰. PSII of a multi-cellular brown alga *Laminaria saccharina* showed emission peak at 687 nm³⁸.

(iii) DCIP photoreduction activity in sporophyte PSII particles was a little lower than in gametophytes²⁰. The different living environment might also affect the photosynthetic efficiency between the two phases of *P. yezoensis*. In natural environment, except for seawater, there is no other barrier that can block light

to irradiate blade gametophytes. In the case of filamentous sporophyte, it lives in the shells that are covered by ceramic glaze, which lowers the photosynthetic efficiency.

(iv) PSII particles of gametophyte and sporophytes showed differences in their polypeptides composition. The gametophyte PSII contained 55 kDa D1 and D2 heterodimer, CP47, CP43, D1, D2, 33 kDa, 20 kDa, 16 kDa and 14 kDa proteins and cyt *c*-550. PSII of sporophytes had almost similar composition, except that 20, 16 and 14 kDa proteins and cyt *c*-550 were not detected and instead cyt *b*-559, and 12 and 102 kDa proteins were found. The 12 kDa protein was reported earlier from PSII of *C. caldarium*¹⁴, and *Synechococcus vulcanus*³⁹. D1/D2 compounds were also reported from other plants⁴⁰⁻⁴². However, to our knowledge, 102 kDa protein from PSII of sporophyte of *P. yezoensis* is new, and not reported earlier. Rubisco activity of gametophytes was about 12-times higher than the sporophytes, suggesting their ways of carbon fixation were different (data not shown).

(v) Oxygen evolution rate of the samples was similar to that of cyanobacterium *Mastigocladus laminosus*²². In this study, we observed that anionic SDS was more effective than Triton-X 100, OG as well as DIG to solubilize *P. yezoensis* sporophyte thylakoid membranes. This observation was in agreement with our previous study in *P. yezoensis* gametophytes thylakoid membranes^{20,21}.

Although the structure of PSII from different photosynthetic organisms was similar, the composition of extrinsic proteins differed, possibly due to evolution of the chloroplasts⁴². PSII of a red alga *C. caldarium* was found to contain four extrinsic proteins □ 33 kDa, 20 kDa, 12 kDa and cyt *c*-550¹⁴⁻¹⁶, whereas PSII from *S. vulcanus*⁴⁰ had all these polypeptides, except 20 kDa protein. PSII of glaucophyte in *Cyanophora paradoxa* also contained cyt *c*-550, but not the 12 kDa protein^{43,44}. However, molecular organization of PSII core complexes from marine brown alga *Laminaria saccharina* was reported to be similar to that of higher plants and cyanobacteria³⁸. In this study, 33 kDa protein, cyt *b*-559, 12 kDa protein, 102 kDa protein were identified as extrinsic proteins of active PSII particles from the sporophyte of *P. yezoensis*. In conclusion, the evidences indicated differences of PSII extrinsic proteins, spectral properties in the two phases of *P. yezoensis*.

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