ISSN 1672-9145 Acta Biochimica et Biophysica Sinica 2004, **36**(11): 780–785 **CN** 31-1940/Q

Short Communication

Isolation and Characterization of Photosystem II of *Porphyra yezoensis* Ueda

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Abstract The thylakoid membranes were isolated and purified from gametophyte of *Porphyra yezoensis* Ueda (*P. yezoensis*) by sucrose density gradient ultracentrifugation. After *P. yezoensis* gametophyte thylakoid membranes were solubilized with SDS, the photosystem II (PSII) particles were isolated and purified. The activity of PSII particles was determined with DCIP (2,6-dichloroindophenol) photoreduction reaction. The composition of purified PSII particles was detected by SDS-PAGE. As a result, seven proteins including 55 kD protein, 47 kD protein, 43 kD protein, 33 kD protein, 31 kD protein, 29 kD protein, and 18 kD protein were found. Compared with PSII particles of higher plants and other algae, they were identified as D1/D2 complex, CP47, CP43, 33 kD protein, D1, D2 and cyt *c*-550 respectively. Besides, other three new proteins of 20 kD, 16 kD and 14 kD respectively were found. Among these extrinsic proteins, the 16 kD and 14 kD proteins had not been reported previously, and the 20 kD protein was found for the first time in multicellular red algae.

Key words red algae; *Porphyra yezoensis* Ueda; photosystem II; polypeptide composition; extrinsic protein

Photosystems include photosystem I (PSI) and photosystem II (PSII). PSII, which is involved in water oxidation, oxygen evolution, hydrogen generation using absorbed light photons, is a multisubunit pigment-protein complex and comprises over twenty identified polypeptides, most of which are membrane proteins [1–3]. The extrinsic part of the intact PSII complex of green algae and higher plants is composed of the 33 kD, 23 kD and 17 kD proteins. Among the three proteins, the 33 kD protein is the most important one and is highly conserved from prokaryotic cyanobacteria to eukaryotic photosynthetic organisms. But the 23 kD and 17 kD proteins are replaced by cytochrome (cyt) *c*-550 and a 12 kD protein in cyanobacteria and red algae. Moreover, a new 20 kD protein was found in PSII of *Cyanidium caldarium*, a red freshwater alga [4–6]. Now PSII research attracts more and more scientists' attention.

From the evolutionary view, photosynthesis was formed at a very early stage [7,8]. It seems that PSII has evolved from an ancestor that was homodimeric according to its protein core and contained special chlorophyll as the photo-oxidizable cofactor [9,10]. But some divergences possibly occurred due to evolution of chloroplasts. According to electron crystallography and X-ray map, Barber and Kühlbrandt found the structure of PSII from spinach and cyanobacteria were very similar [11]. However, cyanobacteria had three transmembrane proteins that were not present in the PSII of spinach chloroplasts, while spinach chloroplasts had a single protein that was lacking in cyanobacteria PSII [12].

Although red algae belong to the eukaryotic organisms, their photosystems share many aspects with those of cyanobacteria. Thus, understanding the structure and component of photosystems of red algae is significant in evolutionary biology study. However, the recent studies about red algae PSII were mostly focused on two uni-

Received: July 20, 2004 Accepted: October 8, 2004
This work was supported by the grants from the National Natural Science Foundation of China (No. 30170499, No. 40476059, No. 30250003, and No. 39890390) and the Project of Scientific Innovation of Institute of Oceanology, the Chinese academy of Sciences (2002-2005)
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cellular freshwater red algae, *Porphyridium cruentum* and *C. caldarium*. There were few reports about PSII of the marine multicellular red algae such as *Porphyra* and *Gracilaria*.

In our study, the seaweed, *P. yezoensis*, was chosen as a material to study its PSII composition, instead of other freshwater unicellular red algae.

Materials and Methods

Materials

The gametophyte of *P. yezoensis* was collected from the seashore of Huiquan Bay, Qingdao, China. The gametophyte was washed with filtered seawater for three times, and then dipped in distilled water for 10 min. After that, the gametophyte was dried by filter paper and stored at –20 °C.

Isolation and purification of the thylakoid membranes from the gametophyte of *P. yezoensis*

15 g frozen gametophyte were fragmented in a triturator containing 75 ml cold extract buffer (50 mM Tris, 5 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 2 mM $NaNO_3$, 100 mM sucrose, 0.5 mM K_2HPO_4, pH 7.8) for 30 min and further broken with ultrasonic at 4 °C for 1 h (60 W, 8 s interval), then filtered with 8 layers of cold gauze. The filtrate was centrifuged at 140,000 *g* (Beckman L8-80, Ti-45 rotor) for 1 h at 4 °C. The pellet was the crude thylakoid membrane. Suspended by the cold extract buffer without sucrose, the thylakoid membranes were put on the density gradient consisting of 60%, 50%, 40%, 30%, 20% sucrose (*W*/*V*) in proportions of 1:1:1:1:1. The gradient was centrifuged at 140,000 *g* (Beckman L8-80, Sw-40 rotor) for 4 h at 4 °C. The bands with different colors were dialyzed against the cold extract buffer without sucrose at 4 °C to remove sucrose.

Preparation of PSII particles from the purified thylakoid membranes

The thylakoid membranes purified from the gametophyte of *P. yezoensis* were treated by the Triton X-100/chlorophyll *a* (Chl *a*) mixture at Triton X-100:Chl *a* ratio of 100:1, 50:1, 25:1 (*W/W*), or SDS/Chl *a* at 100:1, 50:1, 25:1 (*W/W*), respectively, in dark with continual stirring at 4 °C for 30 min. The concentration of Chl *a* was determined according to the method described by Kursar and Alberte [13]. After solubilization, the mixture was placed onto the density gradient consisting of 60%, 50%, 40%, 30%, 20%, 15%, 10% sucrose (*W*/*V*) in proportions of 1:1:1:1:1:1:1 with 0.2% detergent, and centrifuged at 140,000 *g* (Beckman L8-80, Sw-40 rotor) for 20 h at 4 °C. Triton X-100, SDS and sucrose were solubilized in the buffer containing 50 mM Tris, 5 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 2 mM $NaNO_3$, 100 mM sucrose, 0.5 mM K_2HPO_4, pH 7.2. The bands with different colors were dialyzed in dark at 4 °C to remove sucrose.

Absorption and fluorescence spectra

Room temperature absorption spectra were recorded by Beckman DU 650 spectrophotometer. Room temperature fluorescence spectra were performed with Hitachi F-4500 fluorescence spectrophotometer.

Determining the activity of photoreduction of DCIP (2,6-dichloroindophenol) of the bands

The rate of photoreduction of DCIP of bands obtained by the sucrose density gradient centrifugation was measured according to the method described by Gounaris and Barber [14], Tang and Satoh [15].

SDS-PAGE

SDS-PAGE was used to examine the polypeptide composition of the PSII particles according to the method describe by Laemmli [16]. Samples were first precipitated with 9 volumes of cold acetone, and then dissolved in equal volume of loading buffer (0.25 M Tris, 5% glycerol, 1% SDS, 0.025% 2-mercaptoethanol). After incubated in boiling water for 5 min, the samples were applied onto the gel. Separating gel was 12% (pH 8.8), and stocking gel was 5% (pH 6.8). After electrophoresis, the gel was stained with 0.0001% Coomassie brilliant blue G250 (containing 4.75% ethanol, 8.5% H_3PO_4) and distained with water for 6 h.

Results

Obtaining the PSII particles after the solubilization of the purified thylakoid membranes

After the first time of sucrose density gradient centrifugation, four bands with different colors were presented in the centrifuge tube. They were called A_1, A_2, A_3 and A_4 (Fig. 1). According to the absorption and fluorescence emission spectrum, A_4 was determined to be phycoerythrin, A_3 was free pigment, and A_1 and A_2 were purified thylakoid membranes and had similar spectrum properties.

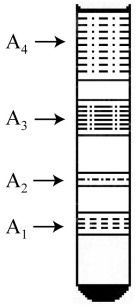

Fig. 1 Results of the first sucrose density gradient ultracentrifugation

A_1 and A_2 were purified thylakoid membranes, A_3 was free pigment, and A_4 was phycoerythrin.

Although A_1 and A_2 were both proved to be thylakoid membranes according to their spectral characteristics, they led to different results under the same condition in the second sucrose density gradient ultracentrifugation as shown in Fig. 2. The results showed that the SDS/Chl *a* mixture with a ration of 25:1 was optimal to solubilize the PSII in purified thylakoid membranes. When the thylakoid membranes were solubilized with SDS:Chl *a* of 25:1, five bands which were labeled as A_1-a, A_1-b, A_1-c, A_1-d, and A_1-e were obtained from A_1; while seven clear bands marked as A_2-a, A_2-b, A_2-c, A_2-d, A_2-e, A_2-f and A_2-g were obtained from A_2. All the three samples treated with Triton X-100 and other two samples solubilized by SDS at the ratio of 50:1 or 100:1 (SDS to Chl *a*) could not lead to good result (data not shown).

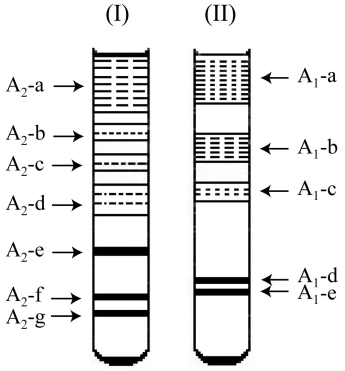

Fig. 2 Results of the second sucrose density gradient ultracentrifugation

(I) was from A_2; (II) came from A_1. Before ultracentrifugation, the samples were treated by SDS. The ratio of SDS versus Chl *a* is 25:1.

The absorption and fluorescence spectra

Fig. 3 showed the absorption spectra of purified thylakoid membranes A_1 (curve 1), A_2 (curve 2) and PSII particles (curve 3). Two prominent peaks at 419 nm and 677 nm were all shown in their spectra. A small peak at 624 nm was also notable. Two shoulders located at 435 nm and 483 nm, which indicated that the samples might contain carotene and the content of pheophytin (Pheo) was thicker than that of Chl. There was no peak of phycoerythrin, which showed that the phycochromoprotein was eliminated thoroughly.

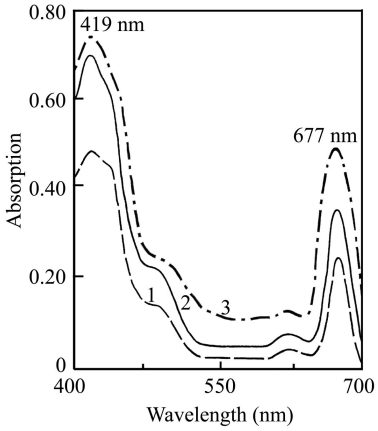

Fig. 3 Absorption spectrum of purified thylakoid membranes

A_1 (curve 1), A_2 (curve 2) and purified PSII particles (curve 3). 419 nm and 677 nm were the prominent peaks; 625 nm was a small peak; two shoulders were 435 nm and 483 nm.

Fig. 4 showed the fluorescence emission spectrum of A_2-g (PSII). At room temperature, the complex exhibited a fluorescence emission maximum at 685 nm (Ex=436 nm).

The activity of DCIP photochemical of PSII

The results of photoreduction of DCIP indicated that only A_2-g was PSII particles, while no photochemical activity could be detected in the bands from A_1. The photochemical activity of PSII with DPC (1,5-diphenylcarbohydrazide) as artificial electron donor was four-time higher than that with H_2O as electron donor (Fig. 5), which indicated that the photochemical activity of the gametophyte *P. yezoensis* PSII particles was quite high.

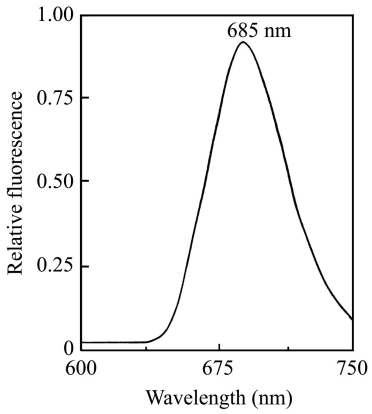

Fig. 4 Fluorescence emission spectrum of A_2-g (PSII) at room temperature (Ex=436 nm)

The excitation wavelength was 436 nm. Its emission peak was at 685 nm.

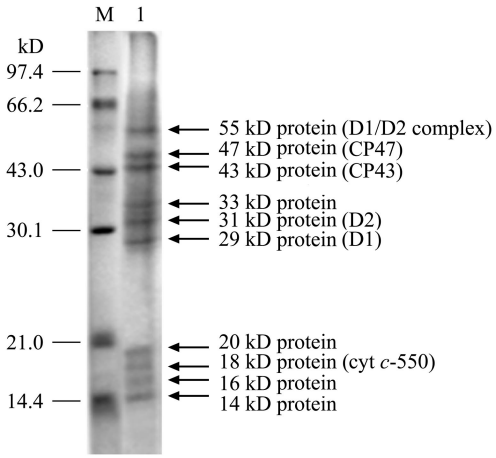

Fig. 6 Composition of A_2-g (PSII) as analyzed by SDS-PAGE

M, molecular marker; 1, A_2-g (PSII). The bands were stained with Coomassie brilliant blue G250.

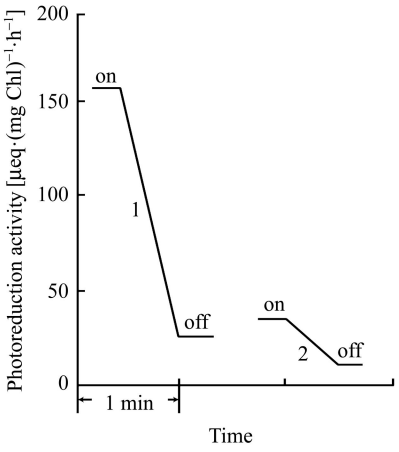

Fig.5 The photoreduction activity of PSII

Curve 1 was the result with DPC (DCIP photoreduction activity of PSII) as electron donor. Curve 2 was the result with H_2O as electron donor (CK).

Composition of the isolated PSII particles

There were 10 protein bands in the SDS-PAGE gel of the gametophyte of *P. yezoensis* PSII particles (Fig. 6), including the 55 kD, 47 kD, 43 kD, 33 kD, 31 kD, 29 kD, 18 kD, 20 kD, 16 kD and 14 kD proteins. According to the results of comparison of PSII particles of higher plants with those of other algae, the former 7 proteins were identified as D1 and D2 complex, CP47, CP43, 33 kD protein, D1, D2, and cyt *c*-550, respectively. Besides, other three new 20 kD protein, 16 kD and 14 kD protein were found; but the 12 kD protein that existed in other red algae and cyanobacteria was not detected.

Discussion

The origin of red algae and their relationships to other eukaryotic organisms have been disputed over one century [17]. Some scientists thought red algae were some of the most primitive eukaryotic algae because of their lack of flagella, basal bodies and resemblance to cyanobacteria in pigment content. But some other scientists thought that red algae had relatively arisen lately according to a phylogenetic tree based on the comparison of 28S rRNA [18]. Therefore, the study on red algal photosystems to clarify the relationships between red algae and cyanobacteria, and higher plants is very significant.

Mild non-ionic detergent is better than ionic detergent to solubilize plant thylakoid membranes [19–22], because ionic detergent can lead to loss of activity of PSII [23, 24]. There was a report that thylakoid membranes of brown algae have natural resistance to the detergent, especially to non-ionic detergent [25,26]. In this paper, we proved that SDS (extensive anionic detergent) was much better than Triton-X 100 (mild neutral detergent) to solubilize *P. yezoensis* gametophyte thylakoid membranes. Therefore, we thought red algae had natural resistance to detergent, too.

The PSII of *P. yezoensis* only had an emission peak at 685 nm, which was a typical PSII emission peak [27]. Two emission peaks, 685 nm and 695 nm, were reported from PSII reaction center of *Spinacia oleracea*, *Pisum sativum* and *Cyanophora paradoxa* [23,28,29]. But Douady *et al.* [30] only found a fluorescence emission peak at 687 nm from PSII of *Laminaria saccharina*. Maybe the

PSII of most seaweeds had no emission peak at 695 nm because we did not find emission peak at 695 nm in the PSII of *P. yezoensis* either. We found D1 and D2 complex in active PSII of *P. yezoensis*. Barber *et al*. [28], Nanba & Satoh [29], Gounaris *et al*. [31] also detected D1 and D2 complex from other plants. Until now, there is no one credible explanation on why D1 and D2 could not be separated from D1/D2 complex even when the samples were solubilized by SDS, which proved once again that the thylakoid membranes of red algae had high natural resistance to detergent.

Although the structure of PSII from different photosynthetic organism was very similar [11], the composition of extrinsic proteins might be different. Enami *et al.* [4–6] identified four extrinsic proteins, the 33 kD, 20 kD, 12 kD protein and cyt *c*-550 in PSII of *C. caldarium*. Whereas, Shen *et al*. [28] only found three extrinsic proteins, the 33 kD protein, 12 kD protein and cyt *c*-550 in the PSII from cyanobacteria. Besides the 33 kD extrinsic protein, Shibata *et al.* [23] also found cyt *c*-550, but they didn't find the 12 kD protein in PSII of glaucocystophyte, *Cyanophora paradoxa*. In this paper, we identified five extrinsic proteins in active PSII from the gametophyte of *P. yezoensis*. They were the 33 kD protein, cyt *c*-550, 20 kD protein, 16 kD and 14 kD proteins. The 20 kD protein was unique because it had been reported only in *C. caldarium*. The 16 kD and 14 kD extrinsic proteins were absent in all other photosynthetic organisms to our knowledge. Like the unique 20 kD protein, the 16 kD protein was a new unique protein found in *P. yezoensis*. As for 14 kD protein, we thought there were three possibilities: (1) it was a new protein of the PSII extrinsic composition of *P. yezoensis*; (2) it was the complex of α-β subunits of cyt *b*-559; (3) it might play a similar role in *P. yezoensis* photosynthesis as the 12 kD protein did in other red algae and cyanobacteria photosynthesis. The present study reported the three new extrinsic proteins found in PSII of *P. yezoensis*, which showed its particular position in life evolutionary process. Because of this, *P. yezoensis* may be probably regarded as a model alga to study the evolution of life in the future. In order to understand the composition and structure of *P. yezoensis* PSII thoroughly, further investigation is necessary.

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Edited by
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