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RESEARCH PAPERS

Isolation and Characterization of the Oxygen-Evolving Photosystem II Complex from the Economical Red Alga *Bangia fusco-purpurea*[1]

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Abstract—The highly pure and active photosystem II (PSII) complex was isolated from *Bangia fusco-purpurea* (Dillw) Lyngb., an important economic red alga in China, through two steps of sucrose density gradient ultracentrifugation and characterized by the room absorption and fluorescence emission spectra, DCIP (2,6-dichloroindophenol) reduction, and oxygen evolution rates. The PSII complex from *B. fusco-purpurea* had the characteristic absorption peaks of chlorophyll (Chl) *a* (436 and 676 nm) and typical fluorescence emission peak at 685 nm (Ex = 436 nm). Moreover, the acquired PSII complex displayed high oxygen evolution (139 μmol O_2/(mg Chl h) in the presence of 2.5 mM 2,6-dimethybenzoqinone as an artificial acceptor and was active in photoreduction of DCIP (2,6-dichloroindophenol) by DPC (1,5-diphenylcarbazide) at 163 U/(mg Chl *a* h). SDS-PAGE also suggested that the purified PSII complex contained four intrinsic proteins (D1, D2, CP43, and CP47) and four extrinsic proteins (33-kD protein, 20-kD protein, cyt *c*-550, and 14-kD protein).

Key words: Bangia fusco-purpurea, photosystem II, fluorescence emission spectra, oxygen evolution.

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INTRODUCTION

Photosynthesis, which is involved in light-induced water oxidation, oxygen evolution, hydrogen generation, and carbon dioxide fixation, is the most important biochemical reaction on Earth [1]. Primary photosynthetic processes in higher plants and algae employ two types of photosystems, namely photosystem I (PSI) and photosystem II (PSII), which are embedded in the thylakoid membrane together with other supramolecular protein units [2]. The PSII complex is a pigment–protein complex, which catalyzes the oxidation of water and the reduction of plastoquinone by utilizing photon energy [3, 4]. PSII consists of four large membrane-intrinsic subunits (D1, D2, CP43, and CP47), several smaller membrane intrinsic and extrinsic subunits [5]. The extrinsic domain of the oxygen-evolving PSII complex of green algae and higher plants is composed of three proteins of 33, 23, and 17 kD [6, 7]. Among the three extrinsic proteins, the 33-kD protein is most important because it is highly conserved from prokaryotic cyanobacterial to eukaryotic photosynthetic organisms. As to other two extrinsic proteins, they are not found in cyanobacteria yet. Instead, two different extrinsic proteins, cyt *c*-550 and 12-kD protein, were found in cyanobacteria [8]. Thus, the oxygen-evolving complex apparently changed extrinsic proteins during its evolution from prokaryotic cyanobacteria to the eukaryotic higher plants while the majority of intrinsic membrane proteins remained almost unchanged.

Red algae are similar to cyanobacteria in many aspects, although they belong to eukaryotic organisms. Firstly, like cyanobacteria, red algae use phycobilisomes as light-harvesting systems [9]. Secondly, the thylakoids of red algae are not stacked but lie equidistantly and singly within chloroplasts while chloroplasts, nucleus, and mitochondria occur in the cells. This indicates that red algae are evolutionarily one of the most primitive eukaryotic algae phylogenetically closely related to the prokaryotic oxygenic cyanobacteria, and the photosynthetic apparatus of red algae appears to represent a transitional state between cyanobacteria and photosynthetic eukaryotes [10]. Therefore, understanding the structure and compo-

[1] This text was submitted by the authors in English.

Abbreviations: Chl—chlorophyll; cyt *c*—cytochome *c*; DCIP—2,6-dichloroindphenol; DPC—1,5-diphenylcarbazide; PSI—photosystem I; PSII—photosystem II; U—units of activity.

nents of photosystems of red algae is an interesting problem in evolutionary biology.

Bangia fusco-purpurea (Rhodophyta) can habit freshwater and seawater and is widespread cultivated for food in the south of China [11]. Many types of oxygen-evolving PSII core complex have been isolated from thylakoid membranes of higher plants, green algae, and cyanobacteria. By comparison, there are only a few reports about PSII of macrophytic red algae and no reports on the PSII complex from the red alga *B. fusco-purpurea*. Moreover, there was no consistent opinion on the composition of extrinsic proteins of red algae. In the present study, the oxygen-evolving PSII core complex was isolated from *B. fusco-purpurea* and characterized after DCIP reduction activity, spectroscopic properties, and polypeptide compositions.

MATERIALS AND METHODS

Alga harvest. The thallus of *Bangia fusco-purpurea* (Dillw) Lyngb. was collected from the cultivar farm of Qingdao, China. The alga was washed with sterile seawater to remove macroscopic contamination and incubated in nutritional seawater, in which 0.1 M $NaNO_3$ and 0.1 M $NH_4H_2PO_4$ were added. Plants were grown at 18°C and illumination in 16 : 8 light/dark cycles with 50 μmol photons/(m^2 s) provided by cool-white fluorescent bulbs . The alga was dipped in distilled water for 10 min before experiment. All reagents were purchased from Shanghai Chemical Plant (China).

Isolation and purification of the thylakoid membranes was performed according to Gao et al. [12] with minor modification. 100 g of alga was fragmented in a triturator containing 500 ml of cold extraction buffer (50 mM Tris–HCl, pH 7.8, 5 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 2 mM $NaNO_3$, 100 mM sucrose, and 0.5 mM K_2HPO_4) and further broken with ultrasonic at 4°C for 0.5 h (50 W, 6-s interval), then, centrifuged at 10000 *g* for 10 min to remove the cell debris. The supernatant was ultracentrifuged at 140000 g (Beckman L8-80, Ti-45 rotor) for 1 h at 4°C. The pellet of ultracentrifugation was suspended in cold extraction buffer without sucrose, then layered on the sucrose density gradient consisting of equal volumes of 60, 50, 40, 30, and 20% (w/v) sucrose and centrifuged at 140000 *g* (Beckman L8-80, Sw-40 rotor) for 3 h at 4°C. Differing in color bands were transferred to different dialysis bags and dialyzed against the cold extraction buffer without sucrose at 4°C for 24 h to remove sucrose.

Preparation of PSII particles from the purified thylakoid membranes. The purified thylakoid membranes of *B. fusco-purpurea* were slowly mixed with 100, 50, and 25 Triton X-100 per 1 Chl *a* and 100, 50, and 25 SDS per 1 Chl *a* (w/w), respectively. After incubation in the dark with gently stirring at 4°C for 30 min, the resultant mixture was loaded on the sucrose density gradient consisting of equal volumes of 60, 50, 40, 30, 20, 15, and 10% (w/v) sucrose with 0.2% detergent and further centrifuged at 140000 *g* (Beckman L8-80, Sw-40 rotor) for 15 h at 4°C. The bands differing in color were dialyzed in the dark at 4°C for 24 h to remove sucrose.

Triton X-100, SDS, and sucrose were solubilized in the buffer containing 50 mM Tris–HCl, pH 7.8, 5 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 2 mM $NaNO_3$, 100 mM sucrose, and 0.5 mM K_2HPO_4. The concentration of Chl *a* was determined spectrophotometrically in 90% acetone extracts according to the method described by Kursar and Alberte [13].

Room absorption and fluorescence spectra. The room absorption spectra (in the range from 400 to 700 nm) were recorded using a UV 757 CRT recording spectrophotometer, and the fluorescence emission spectra were excited at 436 nm and recorded with a Hitachi 850 fluorescence spectrophotometer at room temperature.

Estimation of oxygen evolution rates. The oxygen evolution rates of the samples were measured using a Clark-type electrode (Hansatech Instr., England) with 50 μmol/(m^2 s) illumination at 17°C. The samples (0.01 mg Chl *a*/ml) were suspended in 1.5 ml medium containing 20 mM Mes–NaOH, pH 6.5, 0.3 M sucrose, 20 mM $CaCl_2$, 10 mM $NaHCO_3$, and 10 mM NaCl and supplemented with electron acceptors, 0.05 mM 2,5-dichlorobenzoquinone and 2.5 mM ferricyanide.

The activity of DCIP photoreduction. The DCIP photoreduction activity of every band obtained from the sucrose density gradient ultracentrifugation, either with or without added artificial electron donor DPC, were measured spectrophotometrically at 580 nm (12.9/(mM cm), in a medium containing 40 μM DCIP and 30 mM Mes–NaOH (pH 6.8). The concentration of every band was equivalent to 10 μg Chl *a*/ml.

Polypeptide composition. The samples were first precipitated with 9 volume of cold 90% acetone at –20°C for 1.5 h and then centrifuged at 5000 *g* for 10 min. The pellet was suspended with loading buffer (0.25 M Tris–HCl, 5% glycerol, 1% SDS, and 0.025% 2-mercaptoethanol, pH 7.8). The sample was applied to the gel with prior heating in boiling water for 5 min. The separating gel was 15% (pH 8.8), and the stacking gel was 5% (pH 6.8). Samples were separated using a constant voltage of 60 V at room temperature and visualized by staining with $AgNO_3$. The following proteins were used as molecular weight markers: phosphorylase b (97.4 kD), albumin (66.2 kD), ovalbumin (43.0 kD), carbonic anhydrase (31.0 kD), trypsin inhibitor (20.1 kD), and α-lactalbumin (14.4 kD).

RESULTS

Obtaining the Thylakoid Membranes

After the first sucrose density gradient ultracentrifugation, five main bands with different colors were

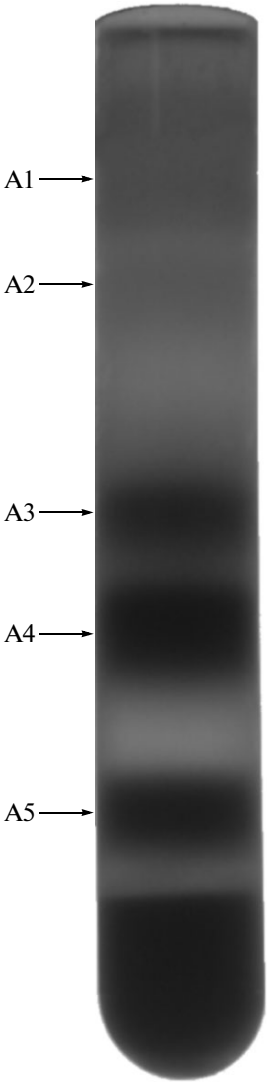

Fig. 1. Results of the first sucrose density gradient ultracentrifugation.

seen in centrifuge tubes (Fig. 1). They were designated A1, A2, A3, A4, and A5. A1 with red color was located in the 0–20% sucrose layer, and A2, A3, A4, and A5 appeared brown located in 20, 30–40, 40–50, and 50–60% sucrose layer, respectively.

Figure 2 showed the absorption peaks of the bands of A1, A2, A3, A4, and A5. Six bands located at 436, 498, 545, 565, 620, and 676 nm were detected in A1. Three peaks at 498, 545, and 565 nm were attributed to R-phycoerythrin, and the other four peaks to phycocyanin (620 nm) and Chl *a* (436 and 676 nm), respectively, indicating that A1 was a free pigment. A2, A3, A4, and A5 had similar absorption peaks, and they had four pronounced peaks at 420, 436, 485, and 676 nm. The peaks at 436 and 676 nm were assigned to Chl *a*, the other two peaks attributed to pheophytin and carotene. These bands lacked the significant absorbance of

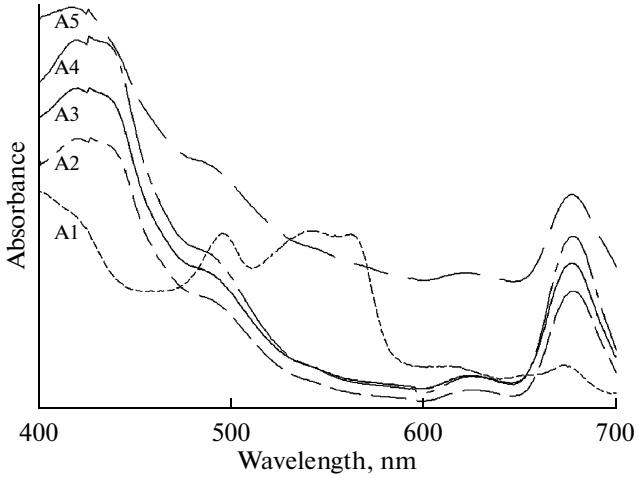

Fig. 2. The room temperature absorption spectra of A1, A2, A3, A4, and A5 bands from the first sucrose density ultracentrifugation.

phycoerythrin, indicating that the sample was free of phycobiliproteins.

Isolation of the PSII

The results of the second ultracentrifugation showed that a detergent Triton X-100 (Triton X-100 : Chl *a* = 25 : 1, w/w) was optimal to solubilize the thylakoid membrane (data not shown). In fact, when the thylakoid membrane was solubilized with SDS at a ratio of 25, 50, or 100 (SDS) to 1 (Chl *a*) or Trition X-100 at the ratio of 50 or 100 (Trition X-100) to 1 (Chl *a*), this could not lead to good results (data not shown). When A2, A3, A4, and A5 were solubilized gently with 25 (Trition X-100) to 1 (Chl *a*) prior to sucrose density gradient centrifugation, no band was acquired from A2, indicating that A2 was free Chl *a*; only one band located in the upper part of the centrifuged tube was obtained from A3, suggesting that A3 might be the aggregation of Chl *a*. Four main bands marked as A4-a, A4-b, A4-c, and A4-d were obtained from A4, and three bands A5-a, A5-b, and A5-c were acquired from A5 (Fig. 3), indicating that A4 and A5 were thylakoid membranes.

Oxygen Evolution Rates and DCIP Reduction Activities

Photochemical activities of DCIP reduction by DPC and oxygen evolution rate of every band acquired from the first and second sucrose density ultracentrifugations, calculated on Chl *a* basis, are shown in the table. Thylakoid membrane A5 evolved oxygen at a rate of 64 μmol O_2/(mg Chl h), and the second sucrose density gradient ultracentrifugation fragment A5-b exhibited a higher activity of 139 μmol O_2/(mg Chl h). Thylakoid membrane A5 was found to be active in photoreduction of DCIP by DPC at 92 U/(mg Chl h).This activity was lower than 163 U/(mg Chl h) of A5-b.

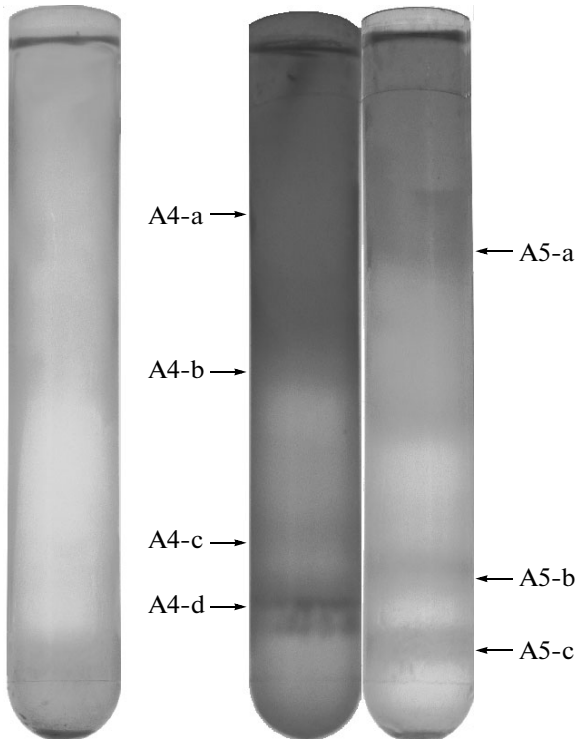

Fig. 3. Results of the second sucrose density gradient ultracentrifugation.
The samples were treated with Triton X-100 (25 Triton X-100 : 1 Chl*a*) before ultracentrifugation.

Oxygen evolution rates and photoreduction activities could not be detected in the bands from A4 and other three bands from A5. Above results indicate that A5-b was PSII particles.

The Aabsorption and Fluorescence Spectra

Room temperature absorption spectra and fluorescence emission spectra (Em = 436 nm) of the purified PSII reaction center complex A5-b are shown in Fig. 4. The absorption spectra showed that purified PSII particles contained pheophytin (420 nm), Chl *a* (436 and 676 nm), and carotene (485 nm). The purified PSII particles exhibited a characteristic PSII emission peak at about 685 nm after excitation at 436 nm.

The oxygen evolution rates and photoreduction activities of thylakoid membranes and PSII isolated from *B. fusco-purpurea*

Sample	Oxygen evolution rate, μmol O_2/(mg Chl h)	Photoreduction activity, U/(mg Chl h)
Thylakoid membrane (A5)	64.0 ± 4.3	92.0 ± 7.1
PSII (A5-b)	139.0 ± 10.4	163.0 ± 7.8

Note: Data represent means ± SD (n = 3).

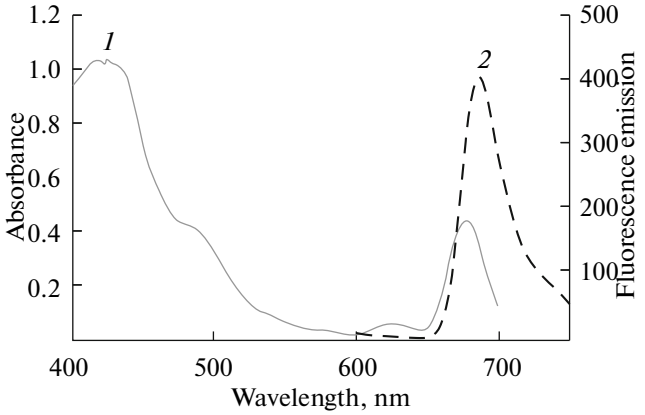

Fig. 4. Room temperature absorption spectra (*1*) and fluorescence emission spectra (*2*) of A5-b (PSII) at room temperature (Ex = 436 nm).

Polypeptide Composition of the Isolated PSII Particles

SDS-PAGE analysis of polypeptide composition of the purified oxygen-evolving PSII particles from the thallus of *B. fusco-purpurea* revealed a high enrichment in this complex. The purified PSII core complex consisted of ten protein bands in the SDS-PAGE gel (Fig. 5), located at 64, 62, 47, 43, 33, 31, 29, 20, 16, and 14 kD. The 64- and 62-kD proteins might be D1/D2 complexes. The bands at 47 and 43 kD were CP 47 and CP 43 apoproteins, respectively. The 33-kD subunit was the manganese stabilizing protein. The bands located at 29 and 31 kD were D1 and D2 proteins, respectively. 20-kD protein was a unique extrinsic protein in red alga, and the band at 16 kD corresponded to cyt *c*-550.

DISCUSSION

Although red algae belong to eukaryotic organisms, their photosystems share many features with those of cyanobacteria. Therefore, studying the red algal photosystems to clarify the relationship between red algae and cyanobacteria and higher plants is quite actual. In this study, the discrete and highly active form of PSII was isolated from *B. fusco-purpurea* thylakoid membranes, using mild detergent conditions and sucrose density gradient ultracentrifugation. Detergents could react readily with the chloroplast system to give fragments of different sizes or even to solubilize chlorophyll [14]. Many kinds of detergents, such as SDS, Triton, digitonin, octyl-β-D-glucopyranoside [15], sodium deoxycholate, Zephiran Chloride, saponin, Tween 20, Duponol C, and Span 80 [14] were used to fragment chloroplasts. Among these detergents, SDS and Triton X-100 were widely used to isolate the PSII from thylakoid membranes. In order to isolated the PSII from *B. fusco-purpurea*, thylakoid membranes were solubilized using two extensively used detergents, SDS and Triton X-100. After treatment with deter-

gents, small fragments of different types were separated by ultracentrifugation. PSII particle could be isolated after solubilization with Triton X-100 and sucrose density gradient ultracentrifugation; they showed the high oxygen evolution rate and DCIP reduction activity. On the other hand, PSII could not be separated after SDS treatment, which indicates that Triton X-100 (mild neutral detergent) was very specific in breaking the hydrophobic interaction between the subunits of PSII core complex and was much better than SDS (an anionic detergent) to solubilize *B. fusco-purpurea* thylakoid membranes. Previous studies also have shown that mild non-ionic detergents were better than ionic detergent to solubilize plant thylakoid membranes [15, 16] because ionic detergent might lead to the loss of PSII activity [15]. However, SDS was much better than Triton X-100 to solubilize *Porphyra yezoensis* gametophyte thylakoid membranes. This discrepancy might be due to the natural stronger resistance to detergent of *P. yezoensis* chloroplasts [16].

The PSII of *B. fusco-purpurea* had only a single emission peak at 685 nm when excited at 436 nm. Similar pattern of emission spectra was acquired from the PSII of *P. yezoensis* and *Laminaria saccharina*, which had only a single emission peak at 685 and 687 nm, respectively [16, 17]. At the same time, the PSII reaction centers of *Spinacia oleracea, Pisum sativum*, and *Cyanophora paradoxa* had two fluorescence emission peaks, a main peak at about 685 nm and a shoulder around 695 nm [18–20]. This indicates that the fluorescence emission peaks of PSII vary in different algae.

The oxygen-evolving PSII complex purified from the red algae *B. fusco-purpurea* was resolved by SDS-PAGE into ten polypeptide bands and visualized by silver staining. Among these, four polypeptides (CP47, CP43 apoproteins, D1, and D2 proteins) have been previously identified and attributed to the reaction center complex. These four intrinsic proteins were similar in the PSII complex of photosynthesizing organisms, whereas the composition of extrinsic proteins varied in different photosynthesizing organisms. Among the photosynthesizing organisms, red algae are one of the most primitive eukaryotic algae phylogentically closely related to the prokaryotic oxygenic cyanobacteria [21]. Consistent with this, PSII from the red algae are partially functional in both the cyanobacterial and higher plant PSII. Previous studies have shown that the extrinsic proteins varied in different red algae. Four extrinsic proteins (33-, 20-, 12-kD proteins, and cyt *c*-550) were identified in PSII of *Cyanidium caldarium* and *Porphyridium cruentum* [22]. Of these proteins, only 33- and 20-kD proteins and cyt *c*-550 were found in *P. yezoensis* PSII [16], while 12-kD protein was absent. Instead, *P. yezoenis* PSII had two different extrinsic proteins, 16- and 14-kD proteins. In this study, the oxygen-evolving PSII complex from the thallus of *B. fusco-purpurea* contained three extrinsic proteins of cyanobacterial type, i.e., the

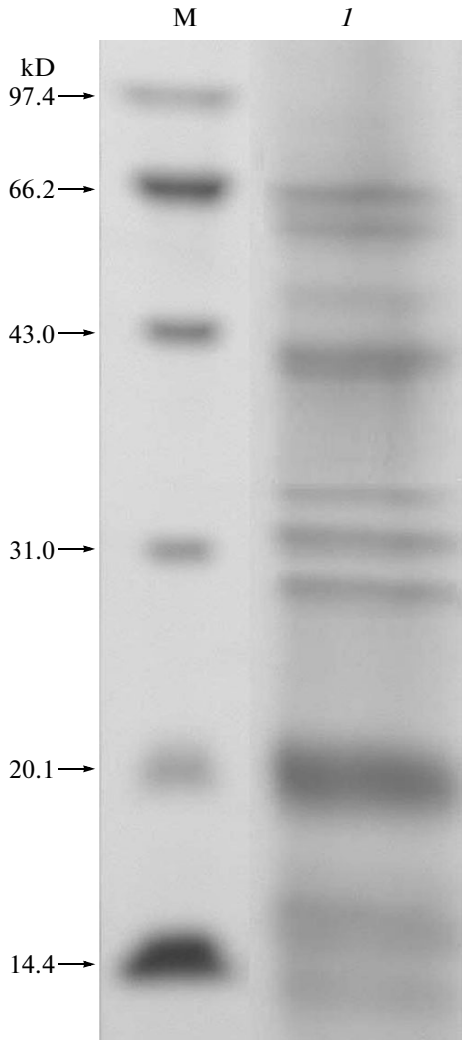

Fig. 5. Polypeptide compositions of A5-b (PSII). M—low-molecular markers. (*1*) PSII from *B. fusco-purpurea*. The bands were stained with $AgNO_3$.

33 kD and cyt *c*-550. In addition to these two proteins, the PSII from *B. fusco-purpurea* contained 20- and 14-kD proteins. The 20-kD protein was unique because it was reported only in red algae. 14- and 16-kD proteins were reported in the PSII from multicellular red alga *P. yezoensis* [16] but not in the unicelluar freshwater red algae *P. cruentum* and *C. caldarium* [23, 24]. These two proteins might be unique proteins of multicellular red algae, which might play similar function as 12-kD protein in photosynthesis of other red algae and cyanobacteria.

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