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Purification and crystallization of oxygen-evolving photosystem II core complex from thermophilic cyanobacteria

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Abstract. This chapter describes the purification and crystallization of oxygen-evolving photosystem II core dimer complex from a thermophilic cyanobacterium *Thermosynechococcus vulcanus*. Procedures used for purification of photosystem II from the cyanobacterium involves cultivation of cells, isolation of thylakoid membranes, purification of crude and pure photosystem II core complexes by detergent solubilization followed by differential centrifugation and column chromatography. The purified core dimer particles were successfully used for crystallization, and the methods and conditions used for crystallization are presented. These purification and crystallization procedures may be applied for another thermophilic cyanobacterium *T. elongatus*.

Keywords: Photosystem II; Oxygen evolution; Crystallization; Membrane proteins; Ion-exchange chromatography

1. Introduction

Highly active oxygen-evolving photosystem II (PSII) core complexes have been purified from various organisms, and used for various biochemical, biophysical, and structural biological studies. The procedures for purification of PSII core complexes from higher plants and a mesophilic cyanobacterium *Synechocystis* sp. PCC 6803 have been described in Chapters 4 and 5 of this volume. Thermophilic cyanobacteria are

unique in that they grow at temperatures much higher (45-60^{o}C) than mesophilic cyanobacteria, so that proteins isolated from thermophilic cyanobacteria are usually much more stable than those from mesophilic cyanobacteria. This has been proven to be a feature important for the structural studies of PSI and PSII, since crystallization of these large membrane-protein complexes has to be performed at medium temperatures for a long period of time. In particular, the oxygen-evolving activity of PSII is rather unstable, and the extrinsic proteins involved in stabilizing the oxygen-evolving complex are usually easy to be released in PSII from mesophilic organisms. In contrast, the extrinsic proteins are found to be tightly associated with PSII purified from thermophilic cyanobacteria (1, 2), and the oxygen-evolving activity of PSII isolated from a thermophilic cyanobacterium *Thermosynechococcus elongatus* has been shown to be stable even after 3 weeks incubation at 20^{o}C (3).

T. vulcanus and *T. elongatus* are two thermophilic cyanobacteria very similar with each other in terms of their gene sequences and cellular growth or photosynthetic characteristics. The two cyanobacteria are isolated from two different Japanese hot springs, and they both grow at 50-60^{o}C. This is among the highest temperatures known so far for photosynthetic organisms to be able to grow. Highly purified and active oxygen-evolving PSII has been obtained from both cyanobacteria, and they were successfully used for crystallization and structure analysis (4-7). So far, the crystal structure of PSII has been solved only for these two cyanobacteria, although PSII from a acido-philic, thermophilic red alga *Cyanidium caldarium* has been crystallized recently (8). Here we describe the purification procedure for PSII core complex from *T. vulcanus*, which involves the culture of cells, isolation of thylakoid membranes and crude PSII, and purification of pure PSII dimer by ion-exchange chromatography. Subsequently, crystallization procedures and conditions for the purified PSII dimers are described. Similar procedures can be applied for the purification and crystallization of PSII from *T. elongatus*.

2. Materials

1. Strain: *Thermosynechococcus vulcanus* (or *T. elongatus*).
2. Growth chamber capable of maintaining temperatures at 50~55^{o}C.
3. High-speed refrigerated centrifuge, ultra-speed refrigerated centrifuge, and angled rotors.
4. Spectrophotometer for determining chlorophyll (chl) concentrations.
5. Brushes for suspending membranes and core particles.

6. Temperature-controlled water bath (38^{o}C).
7. Equipment for column chromatography including pumps and fraction collector, which is capable of making a liner gradient of salt concentration and maintained at a low-temperature chamber or cold room.
8. Chromatography column packed with Q sepharose high-performance (GE Healthcare).
9. Crystallization plates, cover slides and temperature-controlled incubators for crystallization.
10. Microscope for the observation of crystals.
11. Growth media for *T. vulcanus* (see **Note 1**):

    First make 1 L each of the following stock solutions: (i) 45 g KH_2PO_4, 55 g K_2HPO_4; (ii) 100 g $NaNO_3$, 100 g KNO_3, 30 g $MgSO_4{\cdot}7H_2O$; (iii) trace minerals containing 6 g EDTA-2Na, 3.2 g $FeCl_3{\cdot}6H_2O$, 0.132 g $ZnSO_4{\cdot}7H_2O$, 0.376 g $CuSO_4{\cdot}5H_2O$, 4.4 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.003 g $Co(NO_3)_2{\cdot}6H_2O$, 0.8 g $MnCl_2{\cdot}4H_2O$, 6.8 g H_3BO_4. (iv) 60 g $CaCl_2{\cdot}2H_2O$.

    To make the final growth medium, take 5 ml each of the stock solutions (i)-(iii), and 1 ml of stock solution (iv), and dilute to 1 L with distilled water. If the growth medium is to be sterilized, make 1 L of diluted solution containing the stock solutions (i)-(iii) only, and sterilize it. After cooling of the solution, add 1 ml of stock solution (iv) which has been sterilized separately by passing through a 0.22 μm filter. The pH of the final medium should be at 7.0-7.5.
12. Stock solutions and buffers for isolation of thylakoid membranes.

    Stock solutions: 1 M $MgCl_2{\cdot}6H_2O$, 0.5 M EDTA-2Na (pH 8.0), DNase I (1 mg/ml, stored at -20^{o}C), Lysozyme (stored at 4^{o}C).

    Buffers:

    B-1: 40 mM KH_2PO_4-KOH (pH 6.8), 2 L

    B-2: 0.4 M mannitol, 40 mM KH_2PO_4-KOH (pH 6.8), 2 L

    B-3: 30 mM Hepes-NaOH (pH 7.0), 10 mM $MgCl_2{\cdot}6H_2O$, 2 L

    B-4: 25% glycerol, 30 mM Hepes-NaOH (pH 7.0), 10 mM $MgCl_2{\cdot}6H_2O$, 1 L.
13. Solutions and buffers for isolation of crude PSII particles**.**

    Detergent: *N*, *N*-dimethyldodecylamine-*N*-oxide (LDAO) (~30%, Fluka).

    Stock solution: 50% polyethylene glycol (PEG) 1,450 (Sigma), 500 ml.

    B-5: 5% glycerol, 30 mM Hepes-NaOH (pH 7.0), 500 ml.
14. Solutions and buffers for isolation of pure PSII dimer.

    Detergent: 20% n-dodecyl-β-D-maltoside (β-DDM) (from Anatrace Co., store at -20^{o}C).

Column buffer-A: 30 mM Mes-NaOH (pH 6.0), 3 mM $CaCl_2$, 0.03% β-DDM.

Column buffer-B: 30 mM Mes-NaOH (pH 6.0), 3 mM $CaCl_2$, 0.03% β-DDM, 1 M NaCl.

B-6: 30 mM Mes-NaOH (pH 6.0), 20 mM NaCl, 3 mM $CaCl_2$.

15. Buffers for crystallization:

Crystallization buffer C-1: 20 mM Mes-NaOH (pH 6.0), 20 mM NaCl, 10 mM $CaCl_2$, 40 mM $MgSO_4$, 0.03% β-DDM, 20% glycerol, 4-10% PEG 1,450.

Reservoir buffer C-2: 20 mM Mes-NaOH (pH 6.0), 20 mM NaCl, 10 mM $CaCl_2$, 40 mM $MgSO_4$, 0.015% β-DDM, 10% glycerol, 7% PEG 1,450.

3. Methods

The following procedures describe growing of the thermophilic cyanobacterial cells, isolation of thylakoid membranes and crude PSII particles, preparation of purified PSII dimers, and growth of 3-dimensional crystals suitable for X-ray crystallographic analysis.

3.1 Growth of cells and purification of PSII

3.1.1 Growth of cells

T. vulcanus can be grown at temperatures up to 60°C; for laboratory use, cells are typically grown at 50-55°C. Small volumes of liquid culture can be grown in sterilized flasks on a shaker. For accelerating the growth, bubbling with air containing 1-5% CO_2 is recommended. In the beginning of cultivation with a low cell density, the light intensity should be kept weak, for example, around 5-15 μmol photons/m^2s. The light intensity may be increased gradually when the cell density increases; and the final light intensity may reach to 50-100 μmol photons/m^2s. For a larger volume of culture such as 50 L, sterilization may be difficult, and the growth may be carried out without sterilization. Since the cyanobacterium grows at a high temperature, contamination from other bacteria is rather limited. In fact, we routinely grow the cyanobacterium in a 50 L scale without sterilization of the medium. For the growth of the larger volume, continuous circulation of the medium with a strong water pump is required to avoid the sedimentation and adhesion of cells to the walls of the container, and bubbling with CO_2-containing air is strongly recommended.

The doubling time of the cells is typically 6-12 hours, and may be shortened up to 4

hours at optimum conditions. Cells harvested from a 50 L culture at their late logarithmic growth stage typically will give thylakoids of 400-500 mg chl, and this will give rise to 6-12 mg chl of PSII dimers using the purification procedures described below.

3.1.2 Preparation of thylakoid membranes

The cells can be broken either mechanically by glass-beads with a beads-beater or enzymatically with lysozyme followed by an osmotic shock-treatment. Here we describe the method of cell breakage by the enzyme-treatment, together with the procedure for preparation of thylakoid membranes. The following steps 1-4 are carried out at room temperature, and steps after step 4 are performed at 4^{o}C or on ice.

1. Harvest cells from a 50 L culture, and wash them with 1 L of buffer B-1.
2. Suspend the cells in 1 L of buffer B-2, add EDTA from a 500 mM stock solution to a final concentration of 1 mM. Add lysozyme to a final concentration of 1.2 mg/ml. Incubate the solution at 38^{o}C for 2.5 hours with gentle shaking in a water bath.
3. Collect the cells by centrifugation at 12,000 x *g* for 5 min at room temperature, and wash the precipitate with 0.8 L of buffer B-2.
4. Suspend the precipitate with a small volume of B-2 (see **Note 2**). Add 1 L of 20 mM Hepes-NaOH (pH 7.0) which has been pre-heated to 38^{o}C as quick as possible and mix the solution immediately, in order to induce a sufficient osmotic shock to break the cells. Add $MgCl_2$ to a final concentration of 5 mM, and 200 µl of DNase I solution of 1 mg/ml. Mix and incubate for a short time (less than 5 min is enough) to allow the DNase-treatment to proceed. The following steps should be performed at 4^{o}C or on ice.
5. Centrifuge the solution at 15,000 x *g* for 10 min, and wash the precipitate with 1 L of buffer B-3.
6. Suspend the final precipitate to buffer B-4 at a chl concentration of 2-3 mg/ml. This is the thylakoid membranes which can be stored at -80^{o}C.

3.1.3 Preparation of crude PSII particles

Pure PSII dimers are purified in two steps. First, thylakoid membranes are solubilized with LDAO, and crude PSII particles are obtained by several times of differential centrifugations. Subsequently, the crude PSII particles are solubilized by

β-DDM and pure PSII dimers are separated from monomers by ion-exchange column chromatography. These procedures are described in this and the following section. These steps should be carried out at 4°C or on ice under dim light conditions.

1. Take 220 mg chl of thylakoid membranes, wash it with 1 L of buffer B-3 by centrifugation at 9,000 x g for 10 min.
2. Suspend the precipitate with buffer B-5 to a final, overall weight of 110 g.
3. Solubilize the precipitated membranes by adding 0.59 g of LDAO (Fluka, ~30%), and stir gently on ice for 5 min (see **Note 3**).
4. Precipitate the LDAO-treated membranes by centrifugation at 35,000 x g for 1 hour. Suspend the precipitate with buffer B-4 to a final weight of 140 g.
5. Perform the second LDAO-treatment by adding 1.25 g of LDAO, and stir gently on ice for 5 min (see **Note 4**).
6. Remove PSI and other non-PSII components by centrifugation at 100,000 x g for 1 hour. Collect the supernatant, and dilute it two-folds with buffer B-4. Centrifuge at 100,000 x g for another 1 hour to remove residual non-PSII components.
7. Collect the supernatant, add PEG 1,450 to a final concentration of 13%.
8. Centrifuge at 100,000 x g for 30 min. The precipitate yielded is crude PSII particles. Suspend it in B-4 and store at -80°C or liquid nitrogen.

The crude PSII particles obtained above typically have an oxygen-evolving activity of 1,000-2,000 μmoles O_2/mg chl/hr, and a yield of 10-20% based on the chl of the starting thylakoid membranes. A lower oxygen-evolving activity and/or a higher yield suggest contaminations by PSI. In this case, the amount of LDAO added in the second LDAO-treatment step should be reduced by 5-15%. On the other hand, a lower yield suggests an insufficient extraction of PSII by the LDAO solubilization, and the amount of LDAO used in the second LDAO-treatment should be increased by 5-15%.

3.1.4 Preparation of pure PSII dimers and monomers

The crude PSII obtained above contains a large amount of phycobili-proteins as well as some other contaminating proteins such as PSI, and is a mixture of PSII dimer and monomers. This is further purified by ion-exchange column chromatography following solubilization by β-DDM.

1. Solubilize crude PSII in buffer B-4 with 1% β-DDM at 1 mg chl/ml. Stir gently for

30 min on ice in the dark.

2. Filtrate the solubilized crude PSII with a 0.45 μm disc filter, and load the sample onto a Q sepharose high-performance column which has been pre-equilibrated in column buffer-A (see **Note 5**).
3. Wash the column with 17% column buffer-B (e.g., at 170 mM NaCl) at a flow rate of 2-3 ml/min, until the absorbance of the eluate at 280 nm decreases to a sufficiently low and constant level. Most of the phycobili-proteins and residual PSI should be eluted by this washing step.
4. Elute the column with a linear gradient of NaCl of 170-300 mM. A typical elution pattern is shown in Fig .1, where the complexes are eluted in the order of PSII monomers, PSII dimers, and PSI trimers. The amount of PSII dimers is usually much larger than that of PSII monomers or PSI trimers. If the amount of PSII monomers is larger than PSII dimers, some of the PSII dimers may have been monomerized during the preparation of crude PSII by LDAO-treatment, and the conditions for LADO-solubilization should be optimized to minimize the amount of PSII monomers. If the amount of PSI is remarkably increased, it suggests a significant contamination of PSI in the crude PSII particles, and conditions for LDAO-solubilization and subsequent differential centrifugations should be optimized to reduce the contamination by PSI.
5. Collect the fraction of PSII monomers and dimers separately, and dilute them with two volumes of buffer B-6 respectively. Add PEG 1,450 to a final concentration of 13%, and pellet PSII by centrifugation at 100,000 x g for 20 min.
6. Suspend the PSII monomers and dimers with buffer B-6 at a chl concentration as high as possible, and store them in liquid nitrogen. The purified PSII monomers and dimers are stable in B-6; however, for repeated use of the sample, 25% glycerol may be included in the suspending buffer to minimize inactivation during multiple freeze-thawing cycles.

The PSII dimers obtained above bind three extrinsic proteins of PsbO, PsbU, PsbV (cytochrome *c*-550) (Fig. 2), exhibits an oxygen-evolving activity of 2,500-4,000 μmol O_2/mg chl/hour, and has a yield of 2-4% based on the chl from the starting thylakoid membranes. If the activity or yield is much lower, the purification procedures need to be improved. PSII monomers have a slightly lower oxygen-evolving activity and much lower yield than those of PSII dimers.

3.2 Crystallization of purified PSII dimers

Crystals of PSII dimers can be grown with the hanging drop, sitting drop vapor diffusion method, or the batch method, at 20°C. Here we describe the crystallization of PSII dimers by the hanging drop vapor diffusion method (9).

1. Adjust the concentration of PSII dimers to a chl concentration of 4 mg/ml in buffer B-6.
2. To make the crystallization droplets, mix 4~5 µl of PSII dimers with an equal volume of crystallization buffer C-1 (see **Note 6**), and set the droplet to a cover slide whose surface has been siliconized in advance.
3. Set the hanging drop against a reservoir of 0.5 ml containing reservoir buffer C-2, and allow the crystals to grow for up to 1 week at 20°C.
4. Rhombic crystals appear in 3-4 days to 0.5 x 0.3 x 0.05 mm, and reach to a maximum size of 1.0 x 0.7 x 0.1 mm in 1 week (Fig. 3).
5. For X-ray diffraction experiments at 100 K, the crystals are transferred to a cryo-protectant solution containing 25% glycerol, 20 % PEG 1,450 (in addition to the other components contained in the crystallization solution) gradually, and are flash-frozen with a nitrogen gas stream, and stored in liquid nitrogen.

4. Notes

1. BG11 can also be used for growth of the thermophilic cyanobacterium. In addition, a DTN medium has been used for growth of *T. elongatus* (10). The selection of growth media may depend on the cost and easiness of the method with which to make.
2. The volume of buffer B-2 used to suspend the lysozyme-treated thylakoid membranes should be kept as small as possible, in order to ensure the effective osmotic shock to take place in the subsequent step.
3. The amount of LDAO used in the first LDAO-solubilization should not lead to partial solubilization of the thylakoid membranes. That is, there should be no chl present in the supernatant after centrifugation of the solubilized membranes. If there is chl present in the supernatant, the amount of LDAO used for solubilization should be reduced slightly.
4. The amount of LDAO used in the second solubilization step may vary slightly depending on the growth condition of the cells and conditions of the thylakoid

membranes. A suitable amount of LDAO should give rise to a yield of 10-20% of crude PSII based on the chl in the starting thylakoid membranes. Increase the amount of LDAO if the yield is lower than this range, and reduce the amount of LDAO if the yield exceeds 20%.

5. Other columns such as Mono Q (GE Healthcare) or DEAE-650S (TOSHO) can be used. Mono Q is an strong anion exchange column and can be used to purify PSII with a similar washing and eluting salt concentrations (9). If a DEAE-650S column is used, the washing concentration should be reduced to 100 mM NaCl, and a linear gradient of 100-250 mM NaCl should be used for elution.
6. The optimum concentration of precipitation agent PEG 1,450 vary depending on the sample condition, and should be determined for each sample by varying its concentration from around 4.0% to 6.0%, with a 0.5% difference for each step.

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Figure legends

Fig. 1. Elution pattern of β-DDM solubilized crude PSII particles from a Q sepharose high-performance column. Crude PSII particles were prepared with the 2 steps-LDAO solubilization method, and a linear gradient of 170-300 mM NaCl was used to separate PSII monomers and dimers. The eluate was monitored at 280 nm.

Fig. 2. SDS-PAGE analysis of PSII monomers (lane 1) and dimers (lane 2). SDS-PAGE was performed with a 16-22% acrylamide gradient gel (11).

Fig. 3. Crystals of PSII dimers. The dark-green crystals were grown at 20^{o}C for 5 days and have a dimension of 1.0 x 0.5 x 0.1 mm.

Fig. 1.

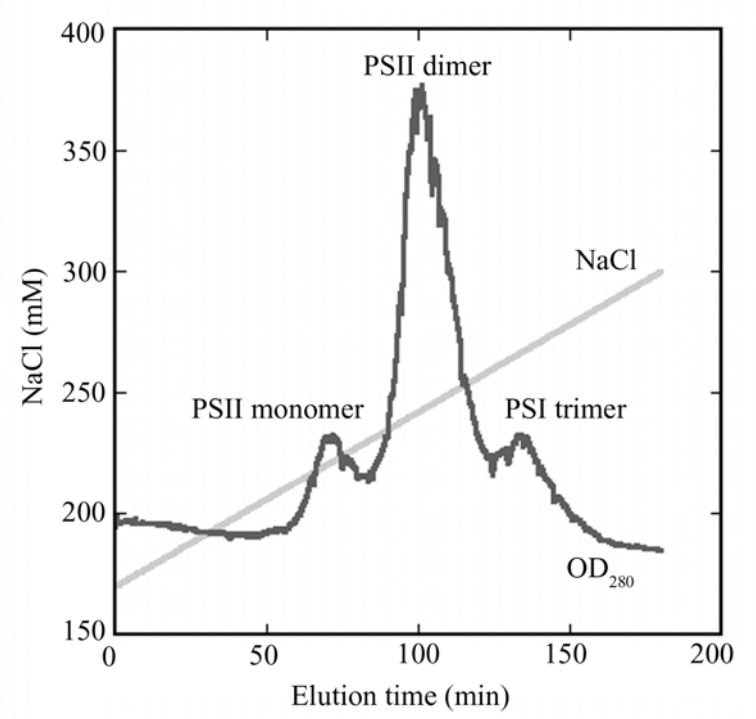
400
350
300
250
200
150
NaCl (mM)
0
50
100
150
200
Elution time (min)
PSII dimer
NaCl
PSII monomer
PSI trimer
OD280

Fig. 2.

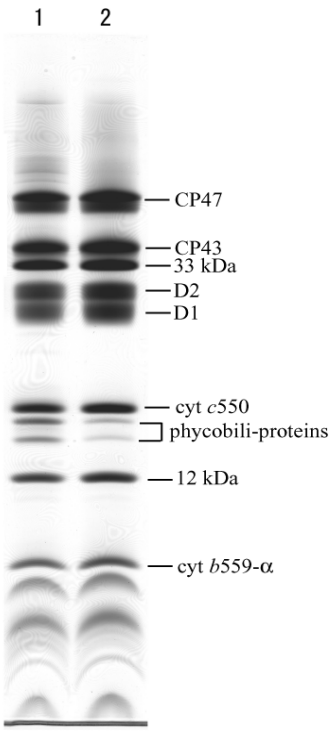
1
2
CP47
CP43
33 kDa
D2
D1
cyt c550
phycobili-proteins
12 kDa
cyt b559-α

Fig. 3.