# Isolation of a highly active PSII-LHCII supercomplex from thylakoid membranes by a direct method

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**Abstract We have developed a simple and novel method to isolate a highly pure and active photosystem (PS) II complex, directly from thylakoid membranes. This complex is a discrete particle and contains all the proteins of the oxygen evolving complex and a set of chlorophyll *a/b* binding proteins. The intactness of both the donor side and the acceptor side has resulted in a very high oxygen evolution activity and therefore offers a superior experimental system to that of PSII enriched membrane fragments in which there is heterogeneity in activities and biochemical composition.**



*Key words:* Oxygen evolution; Photosystem II; Thylakoid membrane

## 1. Introduction

Photosystem II (PSII), a part of the photosynthetic machinery, is located in the thylakoid membranes of cyanobacteria and chloroplasts of plants. This large membrane protein complex contains at least 25 polypeptides [1], including integral membrane proteins and soluble proteins, comprising the core complex. Connected to the PSII core complex, there is a set of chlorophyll *a/b* binding (CAB) proteins, which act as light harvesting antennae. Together, they use light energy to drive the redox reaction that catalyses the oxidation of water and reduction of plastoquinone [2].

Many procedures exist to isolate PSII that usually involve detergent treatments of isolated thylakoid membrane stacks, so-called BBY membranes [3], since the active form of PSII is located in this region [4]. Hankamer et al. [5] recently reported a procedure to isolate a form of PSII which was a discrete particle containing the CAB proteins, CP26 and CP29, and light harvestin complex (LHC) II as well as the CP43, CP47, D1 and D2 core proteins of PSII. Biochemical studies and single particle analysis of electron micrographs showed this PSII-LHCII supercomplex to be a dimer [5,6] with one trimer of LHCII per reaction centre. However, the PSII-LHCII supercomplex lacked the 23- and 16-kDa extrinsic proteins, thus having low oxygen evolving activity [5].

In higher plants and algae the extrinsic proteins of PSII that make up the oxygen evolving complex (OEC), including the 33-, 23- and 16-kDa proteins [1], are located at the lumenal side of the thylakoid membrane [7]. Detergent treatment may disrupt the binding of the 23-kDa, and especially the 16-kDa protein, to PSII. Recently, a more intact PSII-LHCII supercomplex was reported [8], containing the 23-kDa extrinsic protein.

In this work, we describe a new method to isolate the PSII-LHCII supercomplex directly from thylakoid membranes by single step detergent solubilisation, thereby avoiding the necessity for the intermediate preparation of BBY membranes. This complex is highly intact and has extremely high oxygen evolution activity. It therefore offers an improved alternative and more defined preparation of PSII, as compared with BBYs, avoiding heterogeneity and the presence of significant levels of non-PSII proteins.

## 2. Materials and methods

### 2.1. Isolation of thylakoid and BBY membranes

Thylakoid membranes were prepared according to [9] with some modifications. Spinach leaves were ground in 50 mM HEPES pH 7.5, 300 mM sucrose and 5 mM $MgCl_2$. The suspension was passed through a filter and the filtrate was centrifuged at $1000\times g$ for 3 min. The pellet was washed once by centrifugation in the same buffer and then homogenised in 5 mM $MgCl_2$ and diluted 1:1 with 50 mM MES pH 6.0, 400 mM sucrose, 15 mM NaCl and 5 mM $MgCl_2$ followed by 10 min centrifugation at $3000\times g$. The resulting pellet of thylakoid membranes was washed once by centrifugation in 25 mM MES pH 6.0, 10 mM NaCl and 5 mM $MgCl_2$. Thylakoid membranes were suspended and stored in 25 mM MES pH 6.0, 10 mM NaCl, 5 mM $MgCl_2$ and 2 M glycine betaine (MNMβ buffer). BBY membranes were prepared as a reference material essentially according to [9] and stored in MNMβ buffer. High concentrations of glycine betaine were used to stabilise the binding of the OEC proteins on PSII [10].

### 2.2. Sucrose density gradient centrifugation

Sucrose gradients were prepared by freezing and thawing (continuous) or by pouring (discontinuous). The sucrose density gradients prepared by freezing and thawing were made according to [5] with some modifications. 10% (w/v) sucrose, 2 M glycine betaine, 25 mM MES pH 5.7, 10 mM NaCl, 5 mM $CaCl_2$ (MNCβ buffer) and 0.03% (w/v) *n*-dodecyl β-D-maltoside (DM) were mixed and kept in SW-28 ultracentrifuge tubes at −80°C for at least 2 h. The thawing of the frozen mixture was initiated with water at room temperature, covering 2–3 cm of the bottom of the tubes, for 5–10 min. The tubes were then kept in the cold room until the thawing was completed. This freezing and thawing resulted in a gradient of sucrose and betaine. Using SW-28 ultracentrifuge tubes, discontinuous gradient consisted of 4 ml 20% (w/v) sucrose in the bottom, followed by 10–0.5% (w/v) sucrose in 2 ml steps of 0.7% increments. All the solutions contained MNCβ buffer plus 0.03% DM.

### 2.3. Preparation of highly active oxygen evolving PSII complex

Thylakoid membranes (0.5 mg Chl/ml) were disrupted by 6 strokes of a glass homogeniser in the presence of 20 mM DM in MNMβ buffer, a procedure which took about 10–20 s. 700 μl of the homogenate was then loaded onto the sucrose gradients. Centrifugation was carried out at 4°C using a SW-28 rotor (Beckman) at 27 000 rpm for

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*Abbreviations:* CAB proteins, chlorophyll *a/b* binding proteins; Chl, chlorophyll; CP, chlorophyll protein; DM, dodecyl-β-D-maltoside; LHC, light harvesting complex; OEC, oxygen evolving complex; PMSF, phenylmethylsulphonyl fluoride; PSI, photosystem I; PSII, photosystem II


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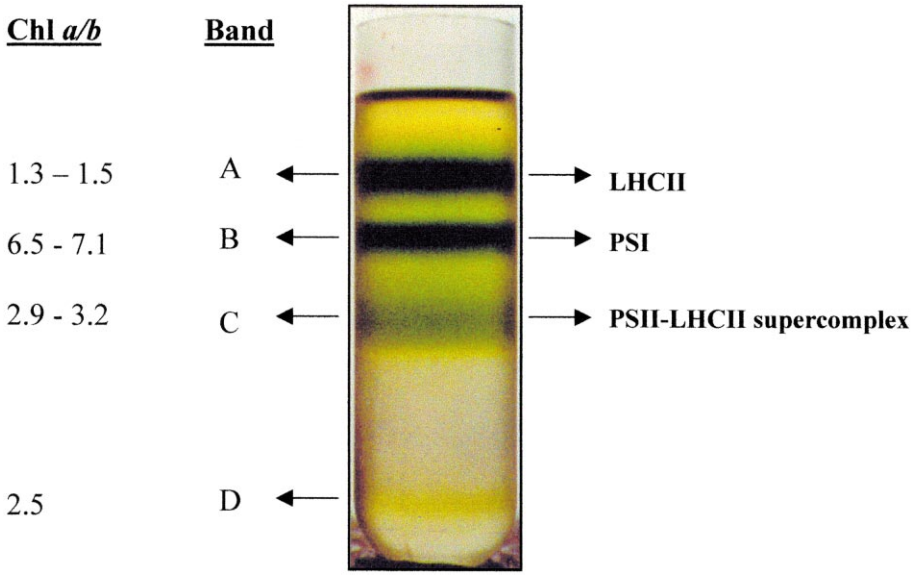


Fig. 1. Sucrose gradient showing different chlorophyll containing bands separated from the solubilised thylakoid membranes after centrifugation.

12–13 h. The resulting chlorophyll containing bands were carefully removed (3–4 ml) using a syringe. 500 μM phenylmethylsulphonyl fluoride (PMSF) was present both during the solubilisation and in the sucrose gradient to inhibit protease activity.

*2.4. Electrophoresis procedures*

The polypeptide composition of the samples was evaluated by SDS-PAGE using 12–22.5% polyacrylamide gel in the presence of 4 M urea in the buffer system of Laemmli [11]. Immunodetection was performed according to an Immuno-Assay kit (Bio-Rad).

*2.5. Oxygen evolution measurements*

The oxygen evolution was measured using a Clark-type oxygen electrode (Hansatech). 1–2 μg chlorophyll of PSII-LHCII supercomplex was added to a medium containing 25 mM MES pH 6.5, 2 M glycine betaine, 25 mM $CaCl_2$, 10 mM $NaHCO_3$ and 10 mM NaCl, and 500 μM phenyl-*p*-benzoquinone as electron acceptor.

*2.6. Pigment analysis*

Chlorophyll levels were determined according to Arnon [12]. Room temperature absorption spectra were measured using a Beckman DU 640 spectrophotometer.

## 3. Results

The aim of this work was to isolate a discrete and highly active form of PSII directly from thylakoid membranes using mild detergent conditions and therefore excluding any intermediate steps such as the preparation of BBY-type membrane fragments. To this end, thylakoid membranes were solubilised gently with DM prior to sucrose density gradient centrifugation. Independent of whether a continuous or discontinuous sucrose gradient was used three main bands, A, B and C, having chlorophyll *a/b* ratios of approximately 1.4, 6.8 and 3.0 respectively, were separated (Fig. 1). There was a fourth very weak band with a Chl *a/b* ratio of 2.5 designated band D in Fig. 1. The protein composition of the three main fractions was determined by SDS-PAGE (Fig. 2). It was clear that the bands A, B and C were enriched in CAB, PSI and PSII proteins, respectively. Band C, in addition to PSII, also contained CAB proteins, which was confirmed by measuring the absorption spectra (Fig. 3). The immunoblotting of the CAB proteins associated with PSII in band C showed the presence of LHCII (Lhcb1 and Lhcb2), CP29 (Lhcb4) and CP26 (Lhcb5) and small amounts of CP24 (Table 2). Two bands were detected by the CP26 antibody in the PSII fraction. However, when the PSII complex was isolated in the presence of PMSF, a protease inhibitor, the lower band disappeared (data not shown). This suggests protease activity on CP26 during the isolation procedure. Furthermore, the isolated PSII complex contained the PsbS protein (see Table 2) but not the LHCII protein, Lhcb3.

Table 1
The oxygen evolution activity of various PSII containing systems

| PSII containing system | $O_2$ evolution (μmol $O_2$ mg $Chl^{-1}$ $h^{-1}$) |
|---|---|
| Thylakoid membranes | 217 ± 83 |
| BBY | 816 ± 74 |
| Band C (PSII-SC) | 1630 ± 240 |

The $O_2$ evolution rate values are the average of four independent determinations.

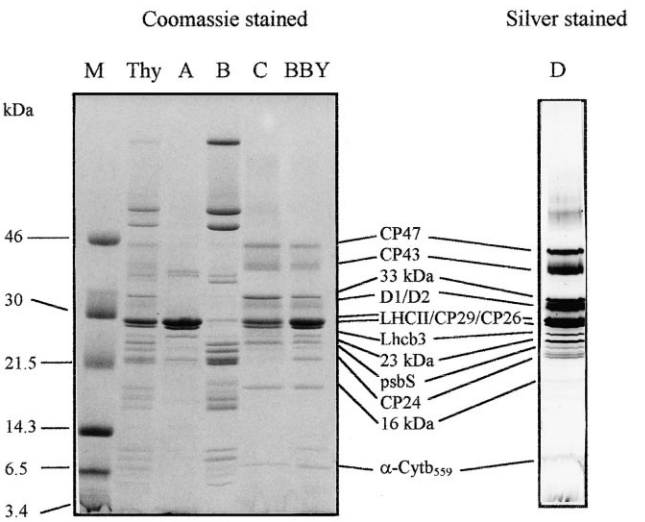


Fig. 2. Polypeptide composition of thylakoid membranes (Thy), sucrose gradient bands A–D and BBY membranes, respectively.

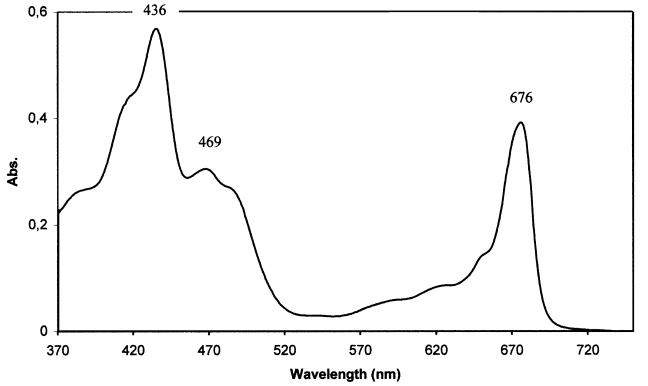


Fig. 3. Room temperature absorption spectra of band C. The peaks are 676 nm, 469 nm and 436 nm.

The above results and the Chl *a*/*b* ratio of band C are in agreement with previous studies of a PSII-LHCII supercomplex isolated from BBYs [5,6,8], indicating band C to be a PSII-LHCII supercomplex. However, in our case this form of the PSII-LHCII supercomplex contained the 16-kDa as well as the 23-kDa and 33-kDa OEC proteins at high levels. Consequently, oxygen evolution measurements showed rates approaching 2000 μmol $O_2$ mg $Chl^{-1}$ $h^{-1}$ (Table 1), suggesting that this supercomplex is highly intact in both donor side and acceptor side function. Moreover, the PSII-LHCII supercomplex was found to be very pure in PSII with minimal contamination by PSI (estimated to be less than 3% by immunoblotting; data not shown).

SDS-PAGE indicated that weak band D is also a pure PSII fraction containing CAB proteins but having a lower chlorophyll *a*/*b* ratio compared to band C. The precise nature and oxygen evolution activity of band D has not been investigated in detail (in part due to low yields) but it does contain the OEC proteins and differs from band C in being enriched in CP24 (Table 2).

## 4. Discussion

In this paper we show that it is possible to isolate a homogeneous and highly active form of PSII directly from thylakoid membranes by mild solubilisation using dodecyl maltoside and sucrose density gradient centrifugation. The preparation contains a complement of CAB proteins as well as all the proteins of the OEC. It is therefore a PSII-LHCII oxygen evolving supercore complex similar to, but considerably more active than that isolated by a more complex procedure involving BBYs [5,6,8]. By excluding all the intermediate purification steps, this PSII-LHCII supercomplex has remained highly intact and active, thus suggesting that this dimeric complex is a natural state of PSII in vivo. This makes the complex a good model for structural studies of PSII. The high activity and intactness of the supercomplex indicate that both the electron donor and acceptor sides are highly preserved from damage and conformational changes due to the procedure adopted for the isolation. It is therefore also an ideal experimental system for biophysical studies.

Table 2
Results from the immunoblotting of band C and band D in comparison with thylakoid and BBY membranes

| | LHCII | Lhcb3 | CP29 | CP26 | CP24 | PsbS |
|---|---|---|---|---|---|---|
| Thy | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| BBY | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| Band C | ++++ | + | ++++ | ++++ | + | ++++ |
| Band D | ++++ | nd | ++++ | ++++ | ++++ | ++++ |

Recently electron microscopy has revealed forms of PSII that are larger than the PSII-LHCII supercomplex [13]. The studies suggest the presence of additional LHCII trimers and in some cases these extra trimers bridge between adjacent supercomplexes [14]. From another study it has been suggested that the attachment of additional LHCII trimers to the supercomplex is via CP24 [16]. It is highly likely therefore that band D is a mixed population of particles observed in [14,15]. This would explain the lower chlorophyll *a*/*b* ratio and enrichment of CP24 in band D compared to bands B and C.

These and other studies [6,13,14,17] are therefore consistent with the idea that the supercomplex that we have isolated in band C is a basic structural unit in the thylakoid membrane, surrounded and interconnected by LHCII trimers. It has been shown previously that the PSII-LHCII supercomplex contains about 100 chlorophylls per reaction centre [5] while the corresponding level for BBYs is about 200–250. Since we observed rates of oxygen evolution from the isolated supercomplex of about 2 times that of BBYs, we can conclude that the form of PSII-LHCII supercomplex that we have isolated directly from spinach thylakoid membranes has maintained full oxygen activity and offers a superior experimental system to that of PSII enriched membrane fragments in which there is heterogeneity in activities and biochemical composition. Moreover, the PSII-LHCII supercomplex is a discrete particle rather than partially delipidated membrane sheets, as is the case with BBYs, and as such provides a framework for locating the relative positions of the major proteins of which it is composed. Recently the procedure has been successfully applied to other plants, including the thylakoid membranes of wild type and PSII mutants of *Chlamydomonas reinhardtii* (F. Morais, S. Eshaghi, P.J. Nixon, B. Andersson and J. Barber, unpublished results).

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