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02 September 2025

Title

One-step isolation and biochemical characterization of a highly active plant PSII monomeric core

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Abstract

We describe a one-step detergent solubilisation protocol for isolating a highly active form of Photosystem II (PSII) from *Pisum sativum* L. Detailed characterization of the preparation showed that the complex was a monomer having no light harvesting proteins attached. This core reaction centre complex had, however, a range of low molecular mass intrinsic proteins as well as the chlorophyll binding proteins CP43 and CP47 and the reaction centre proteins D1 and D2. Of particular note was the presence of a stoichiometric level of PsbW, a low molecular weight protein not present in PSII of cyanobacteria. Despite the high oxygen evolution rate, the core complex did not retain the PsbQ extrinsic protein although there was close to a full complement of PsbO and PsbR and partial level of PsbP. However, reconstitution of PsbP and PsbPQ was possible. The presence of PsbP in absence of LHCII and other chlorophyll a/b binding proteins confirms that LHCII proteins are not a strict requirement for the assembly of this extrinsic polypeptide to the PSII core in contrast with the conclusion of Caffarri et al. (2009).

Keywords Photosystem II, monomer, *Pisum sativum*, PsbP, oxygen evolving activity

Abbreviations

BN-PAGE Blue native polyacrylamide gel electrophoresis; Chl Chlorophyll; DCBQ 2,6-dichlorobenzoquinone; DCMU 3-(3,4-dichloropheny1)-l,l-dimethylurea; EM Electron microscopy; ESI Electrospray ionization; $K_3Fe(CN)_6$ Potassium ferricyanide; LC Liquid chromatography; LHC Light harvesting complex; MALDI-TOF Matrix-assisted laser desorption/ionization-time of flight; MS Mass spectrometry; OEC Oxygen evolving complex; PEG6000 Polyethylene glycol Mr 6000; PSII Photosystem II; SDS-PAGE Sodium dodecyl sulphate polyacrylamide gel electrophoresis; α-DM alpha-Dodecylmaltoside; β-DM beta-Dodecylmaltoside

Introduction

There has been considerable success in obtaining detailed information of the structure of Photosystem II (PSII) isolated from cyanobacteria (Zouni et al. 2001; Kamiya and Shen 2003; Ferreira et al. 2004 ; Loll et al. 2005; Guskov et al. 2009; Shen JR et al. unpublished). The challenge is to extend this degree of structural knowledge to PSII of higher plants and green algae.

There is a number of significant differences between the PSII of cyanobacteria and that of higher plants and green algae. Higher plant PSII contains at least two unique low molecular mass subunits not found in cyanobacteria, the intrinsic PsbW and the extrinsic PsbR and, in the case of the extrinsic proteins which form a part of the Oxygen Evolving Complex (OEC), the PsbP and PsbQ (Bricker and Burnap 2005) are present rather than PsbU and PsbV as in cyanobacteria, although the latter may have similar proteins under some circumstances (Thornton et al. 2004). Other differences concern the outer antenna systems. In higher plants the PSII reaction centre core complex is serviced by an intrinsic light harvesting complex (LHC) binding chlorophyll (Chl) a and Chl b, while in cyanobacteria there are extrinsic phycobilisomes attached to the stromal surface of PSII, where their phycobilines absorb light and transfer the excitons to the reaction centre. This difference in the outer antenna systems suggests significant differences in binding sites for these peripheral pigment containing proteins.

So far the structural picture for PSII of higher plants and green algae is limited to studies using relatively low resolution freeze fracture and transmission electron microscopy (Hankamer et al. 1997a; Staehelin 2003; Dekker and Boekema 2005). At present the highest resolution 3D structures available for higher plant PSII have been obtained at 8 to 9 Å by electron cryo-crystallography for the reaction centre core (Rhee et al. 1997; Rhee et al. 1998; Hankamer et al. 2001) and about 17 Å by analysis of single particles of a LHCII-PSII supercomplex viewed by electron cryo-microscopy (Nield et al. 2000; Nield et al. 2002). The latter has been used to build a more detailed model of the LHCII-PSII supercomplex

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(Nield and Barber 2006) using the crystal structure of the cyanobacterial core dimer (Ferreira et al. 2004) and the isolated higher plant LHCII (Liu et al. 2004; Standfuss et al. 2005), PsbP (Ifuku et al. 2004) and PsbQ (Calderone et al. 2003). Clearly such a model has serious limitations and uncertainties. Therefore, to fully address the architecture of the PSII in higher plants or green algae and to complement biochemical and molecular biological studies of PSII and its OEC proteins (Miyao and Murata 1989; Bricker and Frakel 2003; Meades et al. 2005; Roose et al. 2007; Yi et al. 2008), structural information at high resolution is required.

There have been reports of plant PSII preparations which form 3D crystals (Adir et al. 1992; Fotinou et al. 1993; Piano et al. 2010), but unfortunately they did not give X-ray diffractions at sufficient quality for analysis.

To make progress with the determination of higher plant PSII structure it will be necessary to isolate a fully characterized and highly active and homogeneous preparation. Several methods have been used to prepare PSII reaction centre cores from higher plants which include multiple steps membrane solubilization with rather long exposure to different non-ionic detergents (i.e., Triton X-100, n-octyl beta-D-glucopyranoside, beta-dodecylmaltoside (β-DM), digitonin) followed by a final isolation step through sucrose density centrifugation (Ikeuchi et al. 1985; Bricker et al. 1985; Haag et al. 1990; Hankamer et al. 1997b; Wang et al. 2010), column chromatography (Tang and Satoh 1985; Ghanotakis et al. 1987; Leeuwen et al. 1991) or a combination of these two methods (Satoh and Butler, 1978). A few of these preparations start from digitonin solubilized chloroplasts, whereas the majority relies on grana membrane fractions derived by solubilization of whole intact thylakoids with Triton X-100 (often called BBYs, see Berthold et al. 1981), a detergent suggested to partially destabilize PSII (Yu et al. 1993). Nevertheless, such isolated reaction centre cores, depleted of LHC proteins and generally characterized by the presence of a minimal number of subunits (i.e., CP47, CP43, D2, D1, PsbO and Cyt b559 alfa- and beta- subunits), have provided an excellent system for the study of primary photochemistry and oxygen evolution as well as for structural studies by electron microscopy (EM) (see for review Barber 2003). Single and milder detergent treatment of isolated thylakoids has already been performed by using alpha-dodecylmaltoside (α-DM) and β-DM, yielding a variety of different oligomeric forms of PSII: monomeric PSII and different sized supramolecular LHCII-PSII supercomplexes (Dekker et al. 2002; Bumba et al. 2004, Caffarri et al. 2009) and a discrete LHCII-PSII supercomplex (Eshaghi et al. 1999; Morosinotto et al. 2006) respectively.

In this paper we describe an efficient one-step method to isolate highly active PSII cores directly from thylakoid membranes of peas grown under controlled conditions, by performing only one very short and mild solubilization step directly on stacked thylakoids. The procedure employs low concentration of a single detergent (β-DM) in combination with sucrose density gradient centrifugation in the presence of glycine betaine, avoiding either long multi-step treatments with different non-ionic detergents or the requirement of the BBYs intermediate, as commonly reported in the traditional PSII core isolation procedures. This is an innovative protocol because it allows the isolation of a highly active PSII core

depleted of the light harvesting proteins as is the case for previously reported preparations (Ikeuchi et al. 1985; Tang and Satoh 1985; Hankamer et al. 1997b), but retaining an almost full complement of PsbO and PsbR and a considerable amount of PsbP among the extrinsic OEC polypeptides, and a conspicuous number of detected small intrinsic proteins (i.e., PsbJ, PsbT, PsbI, PsbK, PsbL, PsbF, PsbW, PsbH and PsbE).

Materials and methods

Plant growth conditions

Before sowing, pea (*Pisum sativum* L., var. Palladio nano) seeds were treated as described in Pagliano et al. (2006). Germinated seedlings were transferred to pots and grown hydroponically in Long Ashton nutrient solution (Hewitt 1966) into a growth chamber with 8 h daylight, 20°C, 60% humidity and 150 μmol m^{-2} s^{-1} photons. Leaves from plants grown for three weeks were harvested and used for experiments.

Thylakoids isolation and PSII preparation

Thylakoids membranes from 21 day-old pea leaves were isolated according to Eshaghi et al. (1999) and finally stored in 25 mM MES pH 6.0, 10 mM NaCl, 5 mM MgCl$_2$ and 2 M glycine betaine (MNMβ buffer). Thylakoid membranes at a Chl concentration of 1 mg ml^{-1} were incubated for 1 minute with 20 mM β-DM at 4°C in the dark in the presence of the protease inhibitor phenylmethylsulphonylfluoride (500 μM). After a short centrifugation at 20000 g for 10 minutes at 4°C, 450 μl of the supernatant were loaded onto a 0.1-1 M linear sucrose gradient containing 25 mM MES, pH 5.7, 10 mM NaCl, 5 mM CaCl$_2$, 0.5 M glycine betaine and 0.03% β-DM. This gradient was centrifuged at 39000 rpm for 18 h at 4°C (TH-641 rotor, Thermo Scientific). The third sucrose band, containing PSII, was carefully removed using a syringe and, when necessary, concentrated by membrane filtration with Amicon Ultra 100 kDa cut-off devices (Millipore) and then stored at -80°C.

Spectroscopy and pigment analysis

The concentration of Chl was measured according to Arnon (1949). Absorption spectra of sucrose gradient bands were recorded using a Lambda25 spectrophotometer (Perkin Elmer). Low temperature (77K) fluorescence emission spectra were determined using a FL55 spectrofluorometer (Perkin Elmer), equipped with a red sensitive photomultiplier and a

low temperature device. Samples were excited at 436 nm. The spectral bandwidth was 7.5 nm (excitation) and 5.5 nm (emission). The Chl concentration was approximately 0.5 μg ml^{-1} in 90% glycerol/MNMβ buffer.

Oxygen evolution measurements

The oxygen evolution was measured at 20°C using a Clark-type oxygen electrode (Hansatech) under saturating light intensity (1000 and 5000 μmol m^{-2} s^{-1} photons respectively for thylakoids and PSII). 10 μg Chl of thylakoids (or 5 μg of PSII) were added to 1 ml of a medium made of 25 mM MES pH 6.5, 2 M glycine betaine, 10 mM NaHCO$_3$ and 10 mM NaCl, either in the presence or without 25 mM CaCl$_2$. A mixture of 1 mM potassium ferricyanide (K$_3$Fe(CN)$_6$) and 200 μM 2,6-dichlorobenzoquinone (DCBQ) or 500 μM DCBQ alone were used as electron acceptors for thylakoids and PSII respectively.

Biochemical characterization of the isolated PSII complex

The purity of the PSII preparation was analysed by size-exclusion chromatography on a Jasco HPLC system with a BioSep-SEC-S 3000 (Phenomenex) column. The 20-μl sample injected contained 5 μg of Chl and the profile was monitored at 280 nm. The mobile phase consisting of 20 mM MES pH 6.5, 10 mM MgCl$_2$, 30 mM CaCl$_2$, 0.5 M mannitol and 0.03% β-DM passed through the column at a flow rate of 1 ml min^{-1}. The molecular weight of the complex was determined by using a gel filtration calibration kit (Gel filtration standard, Bio-Rad). Electrophoresis was carried out using either the Laemmli's system (Laemmli 1970) or the Kashino's system (Kashino et al. 2001) in order to resolve the small subunits. In the former, protein bands were resolved on 12.5% polyacrylamide gels in the presence of 5 M urea; in the latter on a linear gradient gel (18-22% acrylamide) containing 6 M urea. Pre-stained protein size markers (Bio-Rad) were used for estimation of apparent size of PSII components. The separated proteins were either stained by Coomassie brilliant blue R-250 or transferred onto nitro-cellulose membrane and immunodetected with specific antisera as previously reported (Pagliano et al. 2006). To estimate the amount of PsbP and other subunits present in the PSII core (ie., PsbO, PsbR and PsbW), quantitative western blot analyses were performed and calculations done in the following way: 1) Standard curves with increasing concentrations of thylakoids (based on Chl level) were generated to test different molar amounts of antibodies towards the reaction centre subunits CP47 and D1, as well as the extrinsic polypeptide PsbP and other PSII subunits, in order to find a range of linear response antigen-antibody with the most similar signal intensity for all the antibodies; 2) In the case of PsbP, five sucrose gradient tubes from five independent experiments were completely fractionated from top to bottom in 33 fractions of equal volume, and a same volume of each fraction was

loaded on a gel, followed by blotting with antibodies towards CP47, D1 and PsbP at the previously determined molar amounts. In this way the signals obtained were not saturated. The sum of the density signals of all the fractions of the sucrose gradient corresponding to the isolated PSII core was calculated for the CP47 and D1 antibodies (referred hereafter to as $\sum$ CP47 and $\sum$ D1 respectively), resulting very similar and therefore taken as representative of the 100% of the isolated PSII core. Analogous sum of density signals was calculated for PsbP (referred hereafter to as $\sum$ PsbP) and then the ratio between $\sum$ PsbP and $\sum$ CP47 was calculated. Similarly the same assessment was conducted for PsbO, PsbR and PsbW. Assuming that in the thylakoid membrane there is a constant ratio of 1:1 between these proteins and CP47 and D1, then the ratios for these subunits within the isolated PSII core could be estimated.

Intact protein complexes were separated onto a Blue Native polyacrylamide gel electrophoresis (BN-PAGE) system according to Schagger and von Jagow (1991) by a linear gradient gel (3.5-15% acrylamide). As molecular mass marker, a mixture of lyophilized standard proteins (Amersham, high molecular weight, GE Healthcare) was used.

Cross-Reconstitution experiment

Spinach BBYs, prepared according to Berthold et al. (1981), were washed with 1 M $CaCl_2$ for removal of the extrinsic proteins according to Bricker (1992). Briefly, spinach BBYs were resuspended at Chl concentration of 0.1 mg ml^{-1} in a buffer composed of 50 mM MES-NaOH, pH 6.0, 10 mM NaCl, 5 mM $MgCl_2$, 0.4 M sucrose supplemented with 1.0 M $CaCl_2$. After 3 h of incubation in the dark on ice, membranes were centrifugated at 40000 g for 30 minutes at 4°C. The supernatant containing the extrinsic proteins PsbO, PsbP and PsbQ was dialyzed against 5 mM Mes/NaOH, pH 5.7 overnight and further concentrated by membrane filtration with Amicon Ultra 10 kDa cut-off devices (Millipore).

For cross-reconstitution, pea PSII cores (100 µg of Chl) were diluted at a Chl concentration of 0.05 mg ml^{-1} in a reconstitution buffer made of 25 mM MES-NaOH, pH 6.0, 10 mM NaCl, 5 mM $CaCl_2$, 0.4 M sucrose and incubated with the spinach extrinsic subunits in a ratio of 1:10 between PSII:OEC for 30 minutes in the dark on ice. After reconstitution, PSII cores were diluted at a Chl concentration of 0.75 µg ml^{-1} in the reconstitution buffer and centrifuged at 47000 g for 2 h at 4°C after addition of 40% PEG6000, and the resulting precipitates finally suspended in a buffer similar to the previous one with the only difference of $CaCl_2$ substituted by $MgCl_2$.

MS analyses

For mass spectrometry (MS) analysis, bands of interest were cut from the gel run according to the Kashino's system and proteins were digested in-gel with trypsin (Roche), as described by Hellmann et al. (1995).

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MS/MS analysis of the digested peptide was performed using a QSTAR XL hybrid quadrupole-TOF instrument (Applied Biosystems) coupled with LC Packings Ultimate nanoflow LC system (Dionex). The tryptic peptides mixture was desalted and concentrated in ZipTipC18 devices (Millipore), and then the eluted mixture was dried in speed vac and reconstituted in 0.1% formic acid/5% acetonitrile in water for the separation in nano-HPLC.

For MALDI-TOF mass spectrometric measurements the isolated PSII complexes (30 μg Chl ml^{-1}) were mixed with an equal volume of a saturated matrix (sinapic acid, Laser Biolabs) solution which consists of 60% acetonitrile and 0.1% trifluoroacetic acid. After drying droplets of sample onto a target plate, MALDI-TOF mass analysis was performed using a Voyager-DE PRO MALDI-TOF mass spectrometer (Applied Biosystems). The instrument was operated in linear mode at 25 kV accelerating voltage and 400 ns ion extraction delay with the nitrogen laser working at 337 nm and 3 Hz as described in Jin and Manabe (2005). One hundred laser flashes were accumulated per spectrum. Internal calibration was performed on the samples premixed with the ProteoMass™ Peptide and Protein MALDI-MS Calibration Kit (MSCAL1, Sigma-Aldrich).

EM analyses

Transmission electron microscopy was performed on material prepared by the droplet method, with 2% uranyl acetate as the negative stain, with a Phillips CM200, at 50000 magnifications.

Results

1.   Isolation of a PSII core complex from *Pisum sativum*

Pea thylakoids, extracted by young pea leaves and stored in a buffer containing 5 mM MgCl$_2$ to maintain granal stacking (Berthold et al. 1981), were usually characterized by a Chl a/b ratio of 3.20-3.30 and oxygen evolution rates around 190 μmol O$_2$ mg Chl^{-1} h^{-1} (either with or without Ca^{2+} and Cl$^-$ in the measurement buffer).

After a very short solubilization of the thylakoids with a low concentration of β-DM, a high percentage of solubilized membranes were separated from the insolubilized material and loaded onto the top of sucrose gradient tubes containing glycine-betaine. After sucrose density gradient centrifugation five main bands, labeled B1-5, were separated (Fig. 1a). The polypeptide composition of each band was assessed by SDS-PAGE (Fig. 1b). It was clear that the bands B1 and B2 were enriched in LHCII subunits in different state of oligomerization, band B3 contained PSII core proteins and bands B4 and B5 corresponded to PSI and PSI-LHCI supercomplexes respectively.

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The PSII core located in B3 had no traces of LHCII and PSI proteins, as confirmed either by spectroscopic analyses, with absorption peaks at 674 and 437 nm (Fig. 1c) and a single fluorescence (at 77K) emission peak at 685 nm (Fig. 1d), or by western blot analyses using antibodies against LHCII and PsaA (Fig. 2).

2.   Integrity of the isolated PSII core

a.   Polypeptide composition and functionality of the basic core complex

The integrity of the PSII core complex isolated from the sucrose density gradient assessed by comparative western blot analyses (Fig. 3a), showed a stoichiometric presence of the CP47, CP43, D2, D1 and the small intrinsic subunit PsbW. On the contrary only a low amount of PsbS was detectable (Fig. 3a).

Compared to other PSII cores depleted of LHCII and isolated by sucrose gradient (Ikeuchi et al. 1985; Haag et al. 1990; Hankamer et al. 1997b; Wang et al. 2010), the pea PSII core complex reported here showed a higher integrity in the poly-peptide composition of the OEC: an almost stoichiometric presence of PsbO and PsbR was confirmed, as well as a detect-able amount of PsbP (Fig. 3b). On the contrary PsbQ was undetectable and present mainly at the very top of the sucrose density centrifugation tube, thus being lost at the beginning of the ultracentrifugation (data not shown). A quantification of the amount of the PsbP extrinsic subunit in the PSII core was performed as described in the Materials and Methods. Comparing the densities of the non-saturating immunological signals of PsbP with those of the reaction centre subunits D1 and CP47 in band B3 (assuming a constant ratio of 1:1 between reaction centre subunits and extrinsics in PSII within the thylakoids), the amount of PsbP bound to the PSII reaction centre core was evaluated as 30%, indicative of a retention of a significant quantity of this subunit during the isolation procedure. Immunoblotting indicated that the remaining PsbP was lost along the sucrose density gradient in lower amount than PsbQ and in a more spread out way (data not shown). The partial binding of PsbP probably contributes to the relatively high light saturated oxygen evolution activity displayed by the isolated PSII core complex, reaching oxygen evolution rates of 1010 ± 42 μmol O_2 mg Chl^{-1} h^{-1} in the presence of an optimal concentration of Ca^{2+} and Cl^- (25 mM $CaCl_2$) in the measurement buffer (and 545 ± 28 μmol O_2 mg Chl^{-1} h^{-1} without $CaCl_2$), values comparable or even much higher than those previously reported for monomeric plant PSII cores, some of which already used for structural studies (Haag et al. 1990; Hankamer et al. 1997b). Moreover our preparation displayed a remarkable sensitivity to 3-(3,4-dichlorophenyl)-l,l-dimethylurea (DCMU), indicating that the Q_B site in the PSII core was not severely damaged. Indeed, in the presence of 10 μM DCMU the PSII core displayed a severe inhibition (70%) of the oxygen evolving activity, suggesting that, in contrast to previous similar PSII preparations which were com-

pletely insensitive to this herbicide (Ikeuchi et al. 1985; Ghanotakis 1987), the mild isolation procedure adopted here is able to largely preserve the PSII core from a severe damage of its acceptor side.

b.   Small subunits content

In Fig. 4 the Coomassie stained gel according to Kashino and colleagues (2001) shows the polypeptide resolution pattern of PSII cores and thylakoid membranes from *Pisum sativum*. The higher resolution of the Kashino's electrophoresis system, especially for the low molecular mass, allowed a far better separation of the PSII polypeptides than the Laemmli's SDS-PAGE shown in Fig. 1b and provided samples for nanoLC-MS/MS analyses.

After in-gel trypsin digestion of the bands, nanoLC ESI MS/MS analysis of the digested peptides revealed the identity of the first 13 bands, confirming that, besides the presence of the reaction centre subunits D1 and D2 and of the inner antenna CP47 and CP43 proteins, other components of the isolated PSII core are: the extrinsic polypeptides PsbO, PsbP, PsbR and Psb27 and the small intrinsic subunits PsbE and PsbH. Table 1 reports the sequences of peptides of the extrinsics and the small intrinsic subunits identified and sequenced by ESI-MS/MS (third column). For each identified protein (fourth column), the protein molecular mass (Mr), calculated from the amino acid sequence in the ExPASy database (fifth column), the accession number of the identified protein and the database in which the protein was found (sixth column) and the percentage of residue identities with the *P. sativum*, when available, or *A. thaliana* homolog, determined with the BLAST program (http://www.expasy.org) and the similarity searching tool Proteomes Fasta (http://www.ebi.ac.uk/fasta33/proteomes.html) (seventh column), are reported.

The total number of low molecular mass proteins identified by MALDI-TOF MS was higher than that detected by nanoLC-ESI-MS/MS. Figure 5 shows all the small subunits identified by MALDI-TOF as components of the isolated PSII cores (from the lower to the higher molecular weight): PsbJ, PsbT, PsbI, PsbK, PsbL, PsbF, PsbW, PsbH and PsbE. A list of all the MALDI-TOF assignments for the small subunits is reported in Table 2, as well as some referenced *m/z* peaks for these proteins when available in the literature. It's noteworthy that the PSII components with the lower molecular weights (i.e., PsbJ, PsbT, PsbI, PsbK, PsbL and PsbF) could only be identified by MALDI-TOF MS and not by nanoLC-ESI-MS/MS after in gel trypsin digestion, probably because of a the higher hydrophobicity of these transmembrane proteins (almost completely embedded in the membrane, due to their short length), lowering the accessibility of trypsin enzyme and the presence in their sequences of a lowered number of tryptic cleavage sites.

c.   Presence of other polypeptides

NanoLC ESI-MS/MS analyses revealed the presence of some other polypeptides, either proteins related to the PSII or contaminants, in the isolated PSII core (Fig. 4 and Table 3). In the first category: the thylakoid lumen protein of 18.3 kDa (TLP18.3) and the thylakoid-bound FtsH protease, both involved in the regulation of the PSII repair cycle and clearly tightly bound to the thylakoid membrane; in the second category: the α and β subunits of the chloroplast CF_1-ATPase, the Ferredoxin-NADP$^+$ Reductase and the Rieske protein of the cytochrome b_6/f complex, all impurities in the PSII preparation. The level of these additional proteins, however, is likely to be very low compared to those of the PSII core. A summary of all proteins identified in the preparation is displayed in Table 3.

d.   Restoration of the OEC proteins of the PSII core by cross-reconstitution

Since the mature PsbO, PsbP and PsbQ proteins are highly conserved in higher plants (Bricker and Burnap 2005), the pea PSII core could be complemented with these proteins by reconstitution with OEC extrinsic proteins isolated from spinach (Fig. 6, lane 3). It is noted that the band of PsbP became somewhat broad and double after reconstitution, the reason for this is the slightly different mobility of spinach and pea PsbP.

This cross-reconstitution allowed the restoration of these OEC polypeptides in pea PSII cores partially missing PsbP and completely PsbQ, generating a more complete and homogeneous PSII preparation. However, their binding to the PSII reaction centre core gave only a partial restoration of its oxygen evolving capability: a 12% improvement of oxygen evolution rates over the native particles when measured in absence of Ca^{2+} and Cl^- and the same rate with saturating $CaCl_2$ concentration (Table 4), indicating that the cross-reconstitution protocol could be further optimized to reduce the Ca^{2+} and Cl^- requirement for maximum activity.

3.   Assessment of the oligomeric form of the isolated PSII core

The oligomeric form of the PSII isolated as band B3 was assessed either by electrophoresis in native conditions or by size-exclusion chromatography. In the first case, thylakoid membranes solubilized with β-DM (according to the procedure used for the sucrose gradient separation) and PSII cores were applied directly to BN-PAGE (Fig. 7a). When pea thylakoids were solubilized with β-DM, a sharp green band corresponding to the PSII monomer was distinguished (340 ± 20 kDa, lane 2), with a molecular mass slightly larger than the purified PSII core complex (320 ± 20 kDa, lane 1) which clearly is also monomeric. The different migration length and the less sharp shape of the band of the purified PSII core could be ascribed either to the sub-stoichiometric association to the core of PsbP and PsbQ (both detected in

comparable amount in the initial and in the solubilized thylakoids, data not shown) or to a decrease in bound lipids corresponding to the increase in concentration of the detergent during the isolation procedure as suggested also by Takahashi et al. (2009). The monomeric state of the isolated PSII core was also confirmed by size-exclusion chromatography. Fig. 7b shows the size exclusion HPLC profile of the PSII particle displaying a single distinct peak of a homogeneous sample eluting at 6.80 min, with molecular mass estimated to be 310 ± 20 kDa.

4.   Structural analyses

With respect to PSII enriched membrane fragments (BBYs), in which there is heterogeneity in activity and biochemical composition, isolated highly pure PSII core complexes offer a superior experimental system suitable as starting material for electron microscopy and single particle analyses and for crystallization trials with the aim to grow 3D crystals of higher plants PSII. A preliminary electron microscopy study using negative stain showed that the PSII preparation was homogeneous and confirmed that the isolated complex was monomeric.

Discussion

In this paper we describe the isolation of a PSII monomeric core complex by a one-step detergent solubilization of thylakoids membranes of *Pisum sativum* (pea) plants, grown under precise environmental conditions and harvested in a consistent way. Such care was also required for the isolation of PSI from peas for successful crystallization studies (Amunts et al. 2005). Following solubilization of thylakoids with 20 mM β-DM in the presence of 2 M glycine betaine, we found that almost all PSII was located in a single band (B3) after sucrose density centrifugation. Optical absorption spectroscopy, 77K fluorescence spectroscopy, SDS-PAGE and immunoblot analyses confirmed that B3 was highly enriched in PSII and established that there was no LHCII present or contamination by PSI. This PSII complex was highly active in light induced oxygen production under optimal conditions. The composition of the complex was further investigated using high resolution gel electrophoresis (Kashino et al. 2001) and mass spectrometry (MALDI-TOF MS, nanoLC-ESI MS/MS), identifying not only the reaction centre proteins (D1 and D2), the inner antenna proteins (CP47 and CP43) and α- and β- cytochrome b559 subunits, but also low molecular mass intrinsic proteins (PsbH, PsbI, PsbJ, PsbK, PsbL, PsbT, PsbW). Traces of PsbS, Psb27, TLP18.3 and FtsH proteins were also detected.
Of the extrinsic OEC proteins PsbO, PsbP and PsbR were present, although PsbP was non-stoichiometric in contrast to PsbO and PsbR. We detected no PsbQ in the preparation, but were able to reconstitute this OEC subunit and also PsbP, using proteins isolated from spinach with some benefit for functional activity.

Perhaps the most surprising outcome of the mild isolation procedure described here was that the isolated PSII was almost exclusively in the form of a monomeric core complex (only traces of a pellet of LHCII-PSII supercomplex was present at the bottom of the sucrose gradient tube). Over the years, and based on extensive biochemical and structural analyses, it has been concluded that PSII is mainly present in the granal regions of the chloroplast thylakoids of higher plants and green algae, where it forms a dimeric core complex with LHCII attached to its periphery (Boekema et al. 1995; Dekker and Boekema 2005). Monomeric PSII is found in the stromal thylakoids, where it is likely to be located transiently as part of the repair-assembly cycle which is characteristic of this photosystem (Aro et al. 2005).

A dimeric organization of PSII in cyanobacteria is also generally accepted and this is the oligomeric state which has been used for most X-ray crystallographic studies (Zouni et al. 2001; Ferreira et al. 2004; Loll et al. 2005; Kern et al. 2005; Guskov et al. 2009). Nevertheless, monomeric PSII is also present after detergent solubilization of cyanobacterial thylakoids (Zouni et al. 2001; Kern et al. 2005; Mamedov et al. 2007) and very recently a crystal structure of this form of PSII has been reported (Broser et al. 2010).

However, it has been argued that the monomeric form of cyanobacterial PSII is the native state of the complex *in vivo* and that dimerization is a consequence of the isolation procedure (Watanabe et al. 2009). In this study, it was shown that with low β-DM concentrations a monomeric form of PSII was isolated, while treatment with higher β-DM concentrations caused its dimerization. So it has been argued that the detergent was inducing a dimerization and that this oligomeric state is an artifact (Takahashi et al. 2009). Whether this is a legitimate conclusion is unclear, but it is a difficult argument to apply to higher plants and green algae, where EM studies have clearly identified that supercomplexes binding LHCII and other Chl a/b binding proteins (CP24, CP26 and CP29) have a dimeric PSII core (Nield et al. 2000; Dekker and Boekema 2005). This dimeric organization is readily observed in membrane fragments (Boekema et al. 1999; Morosinotto et al. 2010) as well as in the isolated LHCII-PSII supercomplex (Nield et al. 2000). It is therefore difficult to reconcile this dimeric state of PSII in higher plants as being an artefact due to detergent treatment. It is for this reason that our results are surprising. The solubilization was conducted in the presence of sufficient salts (5 mM $MgCl_2$ and 10 mM NaCl) to keep the grana thylakoids in their stacked configuration and was almost complete with only about 5% of total Chl remaining in the pellet after the initial centrifugation.

In our higher plant monomeric core we detected the PsbL and PsbT small subunits. These proteins together with PsbM were suggested to be required for the dimeric organization of PSII in cyanobacteria, where they are located at the monomer-monomer interface (Ferreira et al. 2004; Loll et al. 2005; Kern et al. 2005). However, this idea seems unlikely since all three proteins are present in the crystal structure of the monomeric cyanobacterial PSII (Broser et al. 2010). It has also been claimed that the PsbW subunit, which is not found in cyanobacteria, is required for dimerization of higher

plant PSII and is only found in the dimeric form of the complex (Thidholm et al. 2002). Here we detected this protein in the monomeric PSII at a level equal to that of the reaction centre proteins.

We included glycine betaine in our buffers during the isolation of thylakoids and PSII for the purpose of stabilizing the OEC extrinsic proteins (Murata et al. 1992; Papageorgiou and Murata 1995; Catucci et al. 1998; Wang et al. 2010). This, coupled with the gentle solubilization procedure, did yield a complex with high oxygen evolving rates and binding more-or-less a full complement of the PsbO and PsbR proteins and about 30% PsbP. In contrast, most reported procedures for isolation of PSII core complexes depleted of LHCII from higher plants resulted in a significant loss of OEC extrinsic proteins and none has been reported to bind PsbP or PsbQ. It has been suggested by Caffarri et al. (2009) that the reason for this is that LHCII and other Chl a/b binding proteins must be present for their binding to occur as in the case of the LHCII-PSII supercomplex (Boekema et al. 1998; Nield et al. 2000) and BBY particles (Berthold et al. 1981). The fact that we identified about 30% binding of PsbP to an isolated PSII core complex devoid of Chl a/b binding proteins places doubt on the conclusion made by Caffarri et al. (2009). Moreover, it seems that reconstitution of PSII with PsbP and PsbQ is possible in the absence of the Chl a/b binding proteins although more work is needed to optimize the reconstitution protocol.

It is noteworthy that we detected Psb27, TLP18.3 and FtsH in the PSII preparation. All three proteins have been implicated in the turnover of PSII (Sirpiö et al. 2007; Nixon et al. 2010) and, as such, they would be expected to be associated with a small fraction of the PSII pool and possibly in a relative loose association with the complex. Their presence could reflect the mild solubilization procedure used.

In conclusion we have found that with the detergent treatment reported here, PSII cores can be isolated from higher plants mainly as a monomeric complex and that this monomeric form has high oxygen evolving capacity. The reason for this is unclear, but presumably represents a combination of factors ranging from the growth regime of the plants to the detergent/protein ratio used during solubilization and possibly the presence of a relatively high level of glycine betaine in buffers used for isolation. To address whether thylakoids used for this study contained dimeric PSII, other procedures were explored and the presence of LHCII-PSII supercomplexes was detected with good yields (details to be published in due course), suggesting that the dimeric organization of PSII is the main state in our starting material. Nevertheless the reproducibility of the monomeric preparation reported here could provide a source of material for crystallization trials and detailed structural studies as has been the case for the cyanobacterial equivalent (Broser et al. 2010).

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Figure legends

Fig. 1 PSII core isolation and characterization: (**a**) sucrose density gradient of solubilized pea thylakoids. Chl containing fractions labeled B1-5; (**b**) SDS-PAGE of prestained protein markers Bio-Rad with their apparent Molecular weight (kDa) indicated on the left (*lane 1*), polypeptide composition of sucrose gradient bands B1-5 (22 μl, *lane 2-6*) and thylakoid membranes (4 μg Chl, *lane 7*); (**c**) absorption spectrum at room temperature and (**d**) fluorescence spectrum at low temperature (77K) of band B3

Fig. 2

Western blots with antibodies against LHCII and PsaA: pea PSII (1 μg Chl, *lane 1*) and pea thylakoids (1 μg Chl, *lane 2*)

Fig. 3

Western blots with antibodies against PSII intrinsic (**a**) and extrinsic polypeptides (**b**): pea PSII (1 μg Chl, *lane 1*), pea thylakoids (1 μg Chl, *lane 2*)

Fig. 4

Profiles of protein composition of pea PSII and thylakoids membranes resolved by SDS-PAGE according to Kashino et al. (2001): pea PSII (5 μg Chl, *lane 1*), pea thylakoids (10 μg Chl, *lane 2*), protein standards Bio-Rad (*lane 3*)

Fig. 5

MALDI-TOF mass spectra of small subunits of isolated PSII. Numerals under the names of PSII subunits indicate *m/z* values. The peak of *m/z* at 5734.59 corresponds to Insulin (Sigma) as calibration marker

Fig. 6

Western blot with antibodies against PsbO, PsbP and PsbQ polypeptides: pea thylakoids (1 μg Chl, *lane 1*), pea PSII (1 μg Chl, *lane 2*), cross-reconstituted pea PSII (1 μg Chl, *lane 3*)

Fig. 7 Assessment of the oligomeric form of the isolated pea PSII core: (**a**) BN-PAGE of pea PSII isolated by sucrose gradient (0.8 μg Chl, *lane 1*), solubilized pea thylakoids (3 μg Chl, *lane 2*) and native high molecular weight marker GE Healthcare (*lane 3*); (**b**) Size-exclusion chromatographic elution profile of a pea PSII isolated by sucrose gradient

Table title

Table 1

ESI MS/MS identifications of extrinsic and small intrinsic subunits of isolated PSII

Table 2

MALDI-TOF assignments of small subunits of isolated PSII

Table 3

Subunits composition of PSII core determined by different methods

Table 4

Oxygen evolution of the isolated PSII core and its reconstituted form with the extrinsic proteins measured in a buffer composed of 25 mM MES pH 6.5, 2 M glycine betaine, 10 mM $NaHCO_3$ and 10 mM NaCl, either in the presence or without 25 mM $CaCl_2$. The O_2 evolution rate values are the average of at least four independent determinations

Table 1

Band # (Fig. 4)	precursor ion mass *m/z*	Sequence	Protein	Mr (kDa)	AC number (gi NCBI) reference organism	% identity with *P. sativum* or *A. thaliana* (sequence coverage)
7	482,8001	LTFDEIQSK	PsbO	34,872	gi|131384 (*Pisum sativum*)	100% *P. sativum*, gi|131384 (51%)
	814,4436	GTGTANQCPTIDGGVDSFSFKPGK				
	647,8677	LCLEPTSFTVK				
	1147,6696	FEEKDGIDYAAVTVQLPGGER				
	880,9000	DGIDYAAVTVQLPGGER				
	482,8001	VPFLFTIK				
	1134,1348	QLVASGKPDSFSGEFLVPSYR				
	788,9232	GASTGYDNAVALPAGGR				
	1121,0129	ITLSVTQTKPETGEVIGVFESIQPSDTDLGAK				
9	802,8834	TNTDYLPYNGDGFK	PsbP	28,030	gi|131390 (*Pisum sativum*)	100% *P. sativum*, gi|131390 (27%)
	792,0653	YEDNFDATSNVSVLVQTTDKK				
	786,8836	SITDYGSPEEFLSK				
	628,8350	QYYNISVLTR				
	694,3432	KFVEDTASSFSVA				
11	726,0249	TTIVMDKNDPNVATAVAELR	Psb27	12,0510	gi|255639881 (*Glycine max*)	88% *A.thaliana*, gi|30678611 (22%)
	685,3478	NDPNVATAVAELR				
	875,1061	DMYSALNAVSGHYISFGPTAPIPAK				
12	798,7074	IKTDTPYGTGGGMDLPNGLDASGRK	PsbR	10,2360	gi|33694227 (*Trifolium pratense*)	100% *A. thaliana*, gi|15219268 (37%)
	1084,9941	TDTPYGTGGGMDLPNGLDASGR				
	870,4252	GVYQFVDKYGANVDGYSPIYEPK				
	836,8869	YGANVDGYSPIYEPK				
13	561,7930	SFADIITSIR	PsbE	9,2834	gi|11497544 (*Spinacia oleracea*)	100% *P. sativum*, gi|295136935 (37%)
	477,7682	QGIPLITGR				
	807,7469	QGIPLITGRFDSLEQLDEFSR				
	743,8290	FDSLEQLDEFSR				
	866,9765	TVVGNLLKPLNSEYGK	PsbH	7,7270	gi|27446474 (*Hydrastis canadensis*)	100% *P. sativum*, gi|295137033 (22%)

Table 2

Observed m/z	Assignment	Referenced m/z
3982,07	PsbJ	–
4059,07	PsbT	–
4207,53	PsbI	4195,5 ±0,3 by MALDI, Zheleva et al. 1998 4209,5±0,5 by ESI, Sharma et al. 1997
4282,91	PsbK	4292,1±0,3 by MALDI, Zheleva et al. 1998
4364,03	PsbL	4365,5±0,3 by MALDI, Zheleva et al. 1998
4405,43	PsbF	4394,8±0,4 by ESI, Sharma et al. 1997
5939,79	PsbW	5927,4±0,3 by MALDI, Zheleva et al. 1998
7696,55	PsbH	–
9282,03	PsbE	9283,7±0,8 by ESI, Sharma et al. 1997

Table 3

	Protein	Subunit	Band # (Fig. 4)	Western blot	MALDI TOF/MS	ESI/MS
Extrinsic subunits	PsbO	33 kDa	7	x		x
	PsbP	23 kDa	9	x		x
	PsbR	10 kDa	12	x		x
	Psb27	11 kDa	11			x
	FtsH protease	75 kDa	1			x
	TLP18.3	18.3 kDa	9			x
Intrinsic subunits	PsbA	D1	8	x		x
	PsbB	CP47	4	x		x
	PsbC	CP43	5	x		x
	PsbD	D2	7	x		x
	PsbE	α-cyt b559	13		x	x
	PsbF	β-cyt b559			x	
	PsbH	H protein	13		x	x
	PsbI	I protein			x	
	PsbJ	J protein			x	
	PsbK	K protein			x	
	PsbL	L protein			x	
	PsbS	Lhc-like protein		x		
	PsbT	T protein			x	
	PsbW	W protein		x	x	
Non PSII	AtpA	α-ATPase CF1	2			x
	AtpB	β-ATPase CF1	3			x
	Fnr	Ferredoxin-NADP+ reductase	6			x
	PetC	Cyt b6f	10			x

Table 4

Oxygen evolution ($\mu mol\ O_2\ mg\ Chl^{-1}\ h^{-1}$)

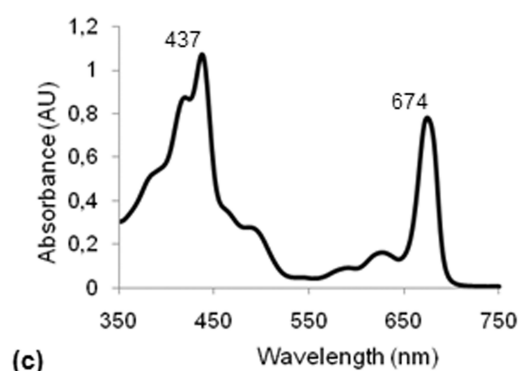|	Isolated PSII	Reconstituted PSII
With 25 mM Ca^{2+}	1010±42	985±40
Without Ca^{2+}	545±28	652±34

Figure 1

Figure 2
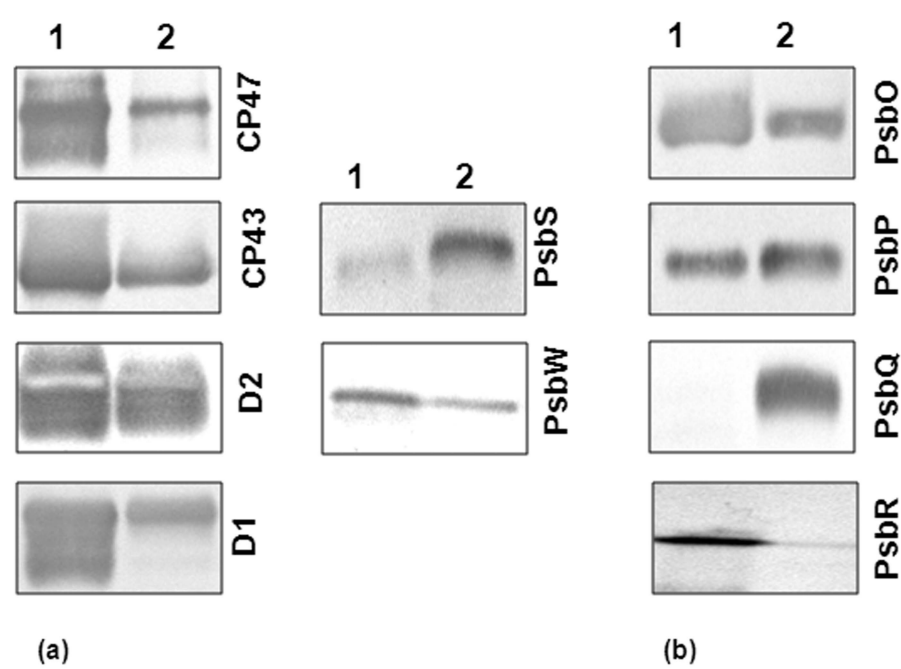
Figure 3

(a)

(b)

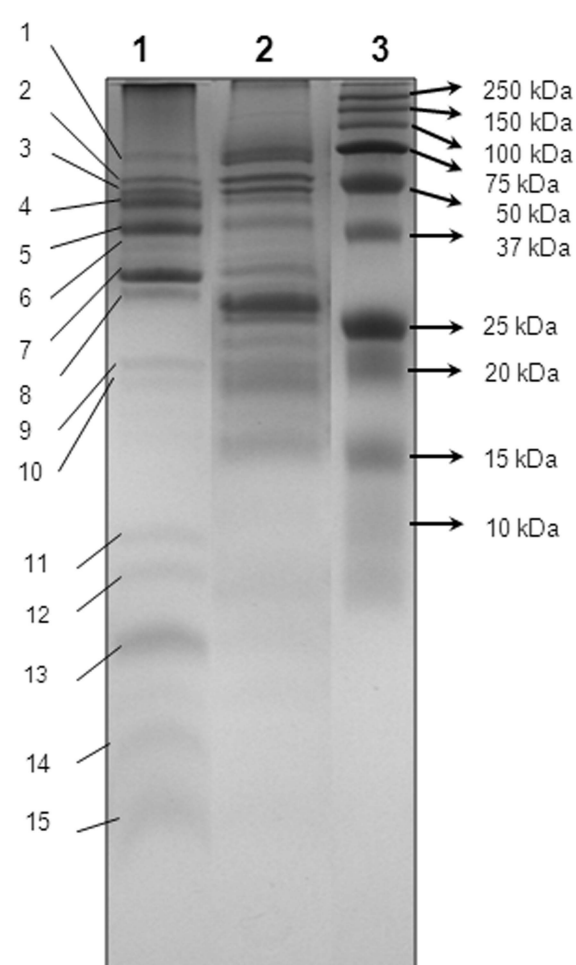Figure 4

250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
15 kDa
10 kDa
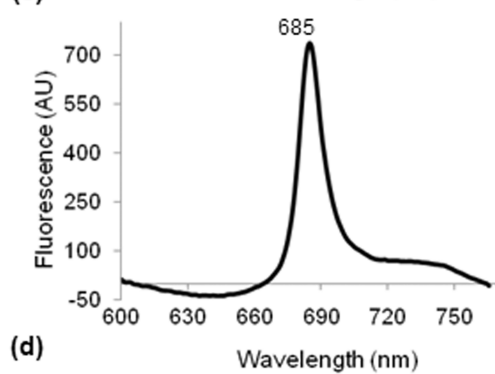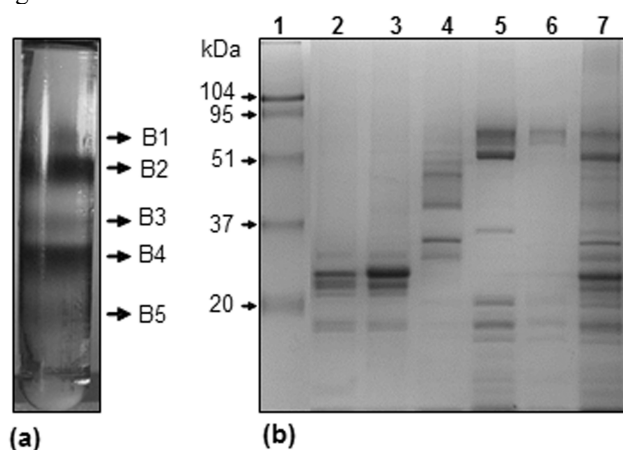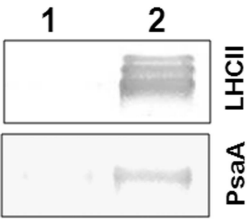
Figure 5

Figure 6

Figure 7

(a)

(b)