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Isolation and characterization of oxygen-evolving photosystem II particles and photosystem II core complex from the filamentous cyanobacterium *Spirulina platensis*

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Abstract

Photosystem (PS) II particles retaining a high rate of O_2 evolution were isolated from the mesophilic filamentous cyanobacterium, *Spirulina platensis*. To achieve high production of PSII complexes in the cells, irradiance from halogen incandescent lamps was used. Disruption of cells by vibration of glass beads proved to be the most suitable procedure for isolation of thylakoid membranes. The selectivity of detergents for PSII particle preparation rose in the order of Triton X-100 < decyl-β-D-glucopyranoside < dodecyldimethyl-aminooxide < *n*-heptyl-β-D-thioglucoside < N-dodecyl-N,N-dimethylammonio-3-propane sulphonate < *n*-octyl-β-thioglycoside < octylglucoside < *n*-dodecyl-β-D-maltoside. The last four detergents yielded extracts, from which pure PSII particles not contaminated by PSI complexes could be obtained by sucrose-gradient centrifugation (20–45%) at the 43% sucrose level. We assumed both the acceptor and donor sides of the isolated *n*-dodecyl-β-D-maltoside (DM) particles to be intact due to high oxygen production by DM particles [1,500 meq(e^-) mol^{-1}(Chl) s^{-1}] achieved in the presence of all artificial acceptors tested. The PSII particle fraction from the sucrose gradient was used with immobilized metal (Cu^{2+}) affinity chromatography (IMAC) for the preparation of the PSII core complex. By washing the column with a MES buffer containing $MgCl_2$ and $CaCl_2$, the phycobiliproteins were stripped off. The PSII core complex was eluted in a buffer containing 1% DM, mannitol, $MgCl_2$, NaCl, $CaCl_2$, and ε-aminocaproic acid. SDS-PAGE of the core complex provided pure bands of D1 and D2 proteins and PsbO protein from thylakoid membrane, which were used to raise polyclonal antibodies in rabbits. These antibodies recognized D1 and D2 not only as monomers of 31 and 32 kDa proteins, but also as heterodimers of D1, D2 corresponding to the band of 66 kDa on SDS-PAGE. This was in contrast to antibodies of synthetic determinants, which reacted only with the monomers of D1 and D2 proteins. These negative reactions against heterodimers of D1, D2 supported the hypothesis that dimeric forms of PSII reaction centre proteins have a C-terminal sequence sterically protected against a reaction with specific antibodies.

Additional key words: antibodies, fluorescence spectra, IMAC chromatography, selectivity of detergents.

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Abbreviations: APC – allophycocyanine; BQ – *p*-benzoquinone; Chl – chlorophyll; CP43, CP 47 – chlorophyll-proteins with R_m 43 and 47 kDa; Cyt – cytochrom; D1, D2 – proteins of PSII reaction centre; DCBQ – 2,6-dichloro-*p*-benzoquinone; DGP – decyl-β-D-glucopyranoside; DM – *n*-dodecyl-β-D-maltoside; DM-PSII – PSII particles isolated upon extraction of TM with DM; DMBQ – 2,6-dimethyl-*p*-benzoquinone; ETR – electron transport rate; FeCy – ferricyanide; HR – Hill reaction; HTG – *n*-heptyl-β-D-thioglucoside; IMAC – immobilized metal affinity chromatography; LDAO – dodecyldimethyl-aminooxide; LT-FER – low temperature (77 K) fluorescence ratio estimated according to the areas under the major bands assumed to originates in the complexes of PSII and PSI; MES – 2-morpholinoethanesulfonic acid; M_r – relative molecular mass; MR – Mehler reaction; OEC – oxygen-evolving complex; OG – octylglucoside; OTG – *n*-octyl-β-thioglycoside; PC – phycocyanine; PS – photosystem; PS-ETR – electron transport through PSII or PSI; $PSQ_{II/I\ ETR}$ – (PSII ETR:PSI ETR); $PSQ_{II/I\ (LT\text{-}FER)}$ – ratio of areas under the bands of 77 K fluorescence emission spectra originated in the components of PSII and PSI; PSQQ – [$PSQ_{II/I\ (ETR)}$:$PSQ_{II/I\ (LT\text{-}FER)}$]; Q_A – primary quinone acceptor of PSII; Q_B – secondary quinone acceptor of PSII; RC – reaction centre; SB12 – N-dodecyl-N,N-dimethylammonio-3-propane sulphonate; ROΔA – redox difference spectroscopy; SDS-PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis; TM – thylakoid membranes.
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Introduction

Selective isolation of high purity photosystem II (PSII) particles from the thylakoids of cyanobacteria is more difficult than a similar procedure starting from chloroplast thylakoids of higher plants. Nevertheless, highly active PSII particles from cyanobacteria have been isolated both for the investigation of photochemical electron transport (Stewart and Bendall 1979, Schatz and Witt 1984, Satoh *et al.* 1995, Kern *et al.* 2005) and as a starting material for isolation of PSII core- and reaction centre complexes (Tang and Diner 1994, Hillmann and Schlodder 1995, Egashira *et al.* 1995, Šetlíková *et al.* 1995). For all these purposes, the thermophilic cyanobacteria and in particular *Synechococcus elongatus* were preferentially used as the source material. Crystals of PSII reaction centre complexes complexes of *Synechococcus* (now called *Thermosynechococcus)* provided the first X-ray structural data at 5 Å resolution (Zouni *et al.* 1998), later medium resolution of 3.8 to 2.9 Å structural maps were presented (Zouni *et al.* 2001, Kamiya and Shen 2003, Ferreira *et al*. 2004, Müh *et al.* 2008, Guskov *et al.* 2009) and recently the crystal structure of oxygen-evolving PSII at a resolution of 1.9 Å from *Thermosynechococcus vulcanus* was reported (Umena *et al.* 2011).

Recently, the transformable cyanobacterium, *Synechocystis* sp. PCC 6803, has been extensively used in genetic studies concerning PSII with the use of directed mutagenesis of genes encoding PSII polypeptides (Vermaas *et al.* 1987, Debus *et al.* 1988, Barbato *et al.* 1999). This stimulated markedly efforts to isolate structurally and functionally well preserved PSII particles from the cells of this organism. Even if some difficulties specific for this strain turned up, several laboratories succeeded in elaborating procedures yielding satisfactory results (Burnap *et al.* 1989, Noren *et al.* 1991, Rögner *et al.* 1990, Bricker *et al.* 1998).

Šetlíková *et al.* (1999) reported a comparison of efficiency of a number of detergents in selective extraction of PSII complexes from *S. elongatus* and the properties of the isolated particles. The main conclusion was that none of the detergents tested yielded particles that would combine high purity (*i.e*. absence of contamination by PSI complexes) with high activity (as a marker of structural and functional integrity). Near-to-optimum results were obtained with either heptylthioglucoside (HTG) or DM. Burnap *et al.* (1989) have recorded similar results when using either octylglucoside (OG) or DM for solubilization of thylakoid membranes from *Synechocystis 6803*. OG extraction was quite selective for PSII but yielded inactive particles. DM solubilized a mixture of PSII and PSI, in which the PSII particles retained high oxygen-evolving activity. An optimal result could be obtained with a mixture of both detergents, in which their best proportion varied slightly according to other conditions or using further purification steps, *e.g*. sucrose-density gradient centrifugation or immobilized Cu^{2+}-affinity chromatography.

Another, as yet unexplained, question is why is the thermophilic *Synechococcus* so advantageous for the isolation of PSII particles of various complexity. It is often proposed that proteins of thermophilic organisms are more stable towards unfavorable impacts of the isolation procedure. We do not feel that this is the decisive factor. Burnap *et al.* (1989) stated that extraction of PSII from the thylakoids of thermophilic cyanobacteria was much more selective than if the same detergents were applied to the mesophilic *Synechocystis*. The difference may be related to the different growth temperatures of the organisms in two ways. The lipid composition of the membranes differs substantially between the thermophilic and mesophilic cells (Fork *et al.* 1979, Wada and Murata 1990, Murata *et al.* 1992). This fact may influence the result of the solubilization in two ways. Either the qualitative difference of the lipid molecules *per se* changes the interaction with the detergents; or, at the laboratory temperature of the extraction, the lipid membranes of the thermophilic cells are more rigid then those of mesophilic cells.

The aim of our work was to test various detergents used for the extraction of PSII particles from the thylakoids of the mesophilic, filamentous cyanobacterium, *S. platensis*. The results were noticeably different from those obtained earlier with the thermophilic *S. elongatus* (Šetlíková *et al*. 1999) and some of them showed parallels to results described for *Synechocystis* elsewhere.

Materials and methods

Growth of the cyanobacteria: Stock cultures of the mesophilic cyanobacterium *S. platensis* (*Arthrospira* sp.), strain Hegewald 1977/229 (Lake Laguna Huacachina, Peru), were obtained from the collection of the Institute of Botany, Academy of Sciences of the Czech Republic, Třeboň (*http://ccala.butbn.cas.cz/index.php*). The cultures were grown at 35°C in the medium (Schlősser 1982). Glass tubes of the inner diameter of 30 mm filled with 0.3 l of suspension were aerated and stirred by a mixture of air with 2% of CO_2. The tubes were immersed in a temperature-controlled water bath with glass walls and irradiated from the side by one of three light sources (*Osram,* Germany):

Light source	Irradiance [μmol m^{-2} s^{-1}]
500 W halogen incandescent lamps *T 838*	500
50 W fluorescent tubes *Lumilux 11*	350
50 W fluorescent tubes *Lumilux 41*	350

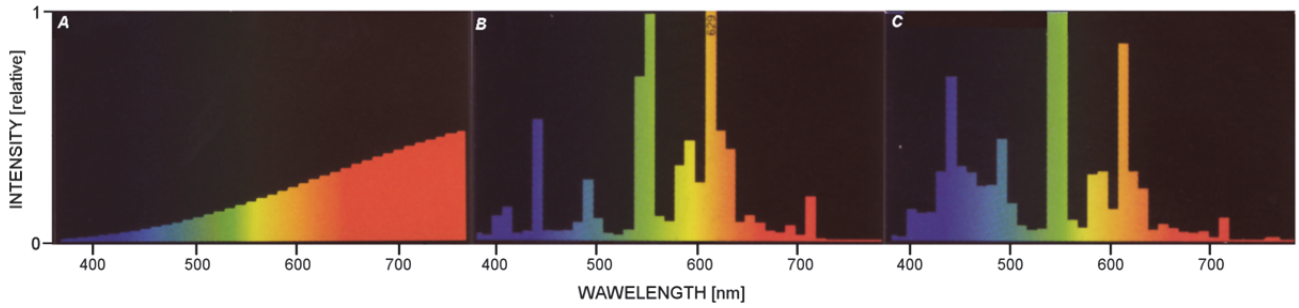

Fig. 1. Relative spectral distribution of radiation sources used for growing the populations of *Spirulina*. *A*: halogen incandescent lamps, T838 (*Osram*, Germany) *B*: fluorescent tubes *Lumilux 41* (*Osram*, Germany); *C*: fluorescent tubes *Lumilux 11* (*Osram*, Germany).

The spectral characteristics of light sources are shown in Fig. 1.

Isolation methods

Thylakoid membranes: The cells were settled by centrifugation (4,000 × *g*, 5 min, 4°C) and washed with buffer A (20 mM MES, 10 mM $MgSO_4$, 15 mM $CaCl_2$, pH 6.5). The sediment of the cells was resuspended in buffer B (20 mM MES, 500 mM mannitol, 35 mM $MgCl_2$, 15 mM NaCl, 15 mM $CaCl_2$, and 1 mM ε-aminocaproic acid, pH 6.5) to the concentration corresponding to chlorophyll (Chl) *a* of 1 mg ml^{-1} for two following two procedures of TM preparation. For disintegration of the cells, three methods were used:

(*1*) Bead-beater (*BIOSPEC,* USA) with glass beads of 150–200 μm in diameter was used for 2 min at 2,000 rpm and at 2°C. Large cells fragments and glass beads were spun down at 4,000 × *g* for 5 min at 4°C. TM were collected from the supernatant by another centrifugation at 24,000 × *g* for 45 min at 4°C. The final sediment of TM was resuspended to the Chl *a* concentration of 1 mg ml^{-1} in the buffer B.

(*2*) French press (*CARVER model M,* USA) was operated at 12 MPa and 2°C. The samples were pressed through three times and the membrane fragments were removed from the supernatant by centrifugation at 4,000 × *g* for 10 min at 2°C. TM in the supernatant were concentrated by centrifugation (24,000 × *g*, 45 min at 4°C) to a suspension containing 1 mg(Chl *a*) ml^{-1}.

(3) The cells were concentrated using centrifugation (4,000 × *g* for 5 min at 2°C) and resuspended in buffer B with enzymes to the concentration of 0.5 mg(Chl *a*) ml^{-1}.

Enzymes (pectinase and hemicellulase C, both from *Aspergillus niger, Sigma,* USA) were dissolved in buffer B to the final concentration of 10^2 U ml^{-1} and lysozyme (from chicken egg, *Serva,* Germany) of 10^6 U ml^{-1}. The incubation was performed at 20°C, in the dark with stirring for 30 min. The resulting suspension was first centrifuged at 3,000 × *g* for 5 min at 2°C to remove large cell fragments. The protoplasts resulting from enzyme treatment were pelleted by centrifugation (6,000 × *g*, 10 min, 2°C) and resuspended in the buffer C (buffer A with 1 mM ε-aminocaproic acid). The osmotic shock in this hypotonic buffer ruptured the protoplasts. TM in the supernatant were settled by a second centrifugation (24,000 × *g*, 30 min, 2°C).

The degree of filament disintegration by all disintegration procedures was measured using a *Fluoval* microscope (*Carl Zeiss*, Jena, Germany). When the optimum degree of filament disintegration (approx. 90%) was exceeded, the photochemical activity of the preparations wass distinctly lowered.

At the end of all three isolation procedures, TM were washed twice with buffer C. This procedure also reduced the quantity of biliproteins still associated with the membranes. After the second washing, the pellets were in all cases resuspended in buffer B containing 10% glycerol to the concentration of 1 mg(Chl *a*) ml^{-1} and stored at –74°C.

PSII particles: TM were resuspended in buffer B to the concentration 1 mg(Chl *a*) ml^{-1} and stirred 30 min in the dark with the detergents under the conditions described in Table 1. (*see* also Fig. 2). The nonsolubilized remnants of TM (usually enriched with PSI particles) were settled by high-speed centrifugation (150,000 × *g* for 2 h at 2°C).

Table 1. Detergent concentration used for the solubilization of thylakoid membranes. All solubilizations were performed at 20°C, in the dark for 30 min with stirring. The density of thylakoid suspensions was adjusted to the final concentration of chlorophyll (Chl) *a* of 1 mg ml^{-1}. SB12, DM, OG, HTG, OTG, DGP, LDAO – *see Abbreviations*.

Detergent	Detergent to Chl ratio [w/w]	Detergent concentration [mol m^{-3}]
SB12	4:1	11.92
DM	10:1	19.58
OG	10:1	34.20
HTG	8:1	27.17
OTG	8:1	25.94
DGP	10:1	31.21
Triton X-100	15:1	24.00
LDAO	8:1	34.87

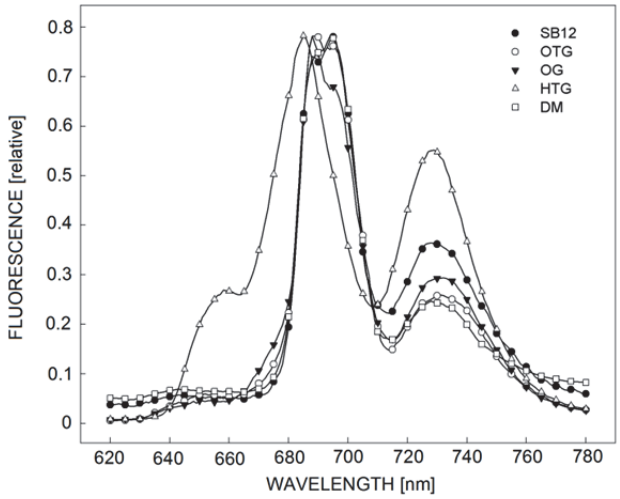

Fig. 2. 77 K fluorescence emission spectra of PSII particles prepared by extraction of thylakoid membranes with different detergents. Excitation at λ = 436 nm. The curves are normalized to the maximum emission between 685 and 696 nm. The shoulder at 660 nm originates most probably from the presence of small amounts of phycocyanin and allophycocyanin that are disconnected from the chlorophyll bearing complexes. SB12, OTG, OG, HTG, DM – *see Abbreviations*.

The supernatant (crude PSII particles) was used for measurement of photochemical PSII and PSI activity and for the subsequent purification by gradient centrifugation. The supernatant was layered on the top of the sucrose-density gradient (20–45% sucrose in buffer B, w/v, with a detergent concentration 10 times lower than that used for solubilization of PSII particles, Table 1) and centrifuged at 160,000 × *g* for 16 h at 4°C. Five layers of sucrose-density gradient were collected and characterized by absorption and fluorescence spectra, denaturing electrophoresis, and immunoblotting. The maximum concentration of PSII particles (according to photochemical activities) was found in 43% sucrose layer.

PSII core complex: For isolation of the PSII core complex, immobilized metal (Cu^{2+}) affinity chromatography (IMAC, Šetlíková *et al.* 1995) was used. The column of the *Chelating Sepharose Fast Flow* was loaded with Cu^{2+} and in the next step with the solubilized PSII particles (about 2 mg of Chl *a*). These become attached by covalent bonds between the amino acids on their surface and the chelated Cu^{2+} ions. Washing the column with 50 ml of buffer D (100 mM MES, 50 mM NaCl, 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, pH 6.5) at a flow-rate of 0.5 ml min^{-1} helped to reduce the quantity of phycobiliproteins and its anchor proteins attached to the core complex. The PSII core complex was eluted from the column with 20 ml of buffer D containing 1% DM at a flow rate of 0.5 ml min^{-1}.

Analytical methods

Photochemical activities: The rates of electron transport were measured in buffer B as oxygen exchange with a Clark-type electrode, *THETA ´90 HC 133* (*Lab. and Industr. Electrochem. Sensors,* Prague, Czech Republic) in a temperature-controlled chamber according to Bartoš *et al.* (1975) on the whole cells, TM, and PSII particles. The electrode was connected to the *OxyMeter* and recorded by *Oxywin* software (both *Photon Systems Instruments,* Brno, Czech Republic). The concentration of Chl *a* in all measured suspensions was 10 μg ml^{-1}. The impermeability of the cell walls and cell membranes of some algae and cyanobacteria to electron donors and acceptors was overcome by using quinones as electron acceptors at a concentration of 4 mM (Warburg and Lűttgens 1944, Koike *et al.* 1992) in measurement of PSII activities (Hill reaction) and 4 mM methylviologen as electron acceptor, 2 mM 3,3'-diaminobenzidine as an electron donor, and 1 μM 3-(3,4-dichlorphenyl)-1,1'-dimethylurea to block PSII activity in measurements of PSI activity as Mehler reaction (Chua 1972). In the case of Hill reaction, the sample in the chamber was flushed with gas mixture (N_2 + 2% CO_2) to reduce the partial pressure of oxygen to about 2 kPa, the electron acceptor was added, and after 2 min of dark adaptation, the saturating irradiance of 3,000 μmol(photon) m^{-2} s^{-1} was applied; output of a 100 W halogen lamp passed through an *RG5 Schott* glass filter (λ > 650 nm) and a neutral filter to adjust its PFD. The PSI activity was measured as the rate of oxygen consumption under the same irradiance without the aeration with gas mixture.

Chl concentration: 50 μl of the samples (TM or Chl protein complexes) were extracted with 5 ml of methanol at room temperature. Absorbance of the filtered extracts was measured at 666 nm and Chl *a* concentration calculated using ε_M = 65.8 (Arnon 1949).

Absorption spectra of cell suspensions, thylakoids and Chl-protein complexes were recorded with a dual wavelength spectrophotometer *Shimadzu UV-3,000* (*Shimadzu*, Japan) in isolation medium for subcellular complexes and using a suspension of light bleached cells in the presence of peracetic acid (40%) as a reference according to Doucha and Kubín (1976).

Fluorescence emission spectra were measured at low temperature (77 K) using the *Fluorolog spectrofluorometer* (*SPEX,* USA) with excitation wavelength of 436 nm and resolution of 0.5 nm. The concentration of the samples corresponded to 2 μg(Chl *a*) ml^{-1}. Data of the emission spectra were deconvoluted into four components on the assumption that each band has a Gaussian distribution (Table 2). A curve fitting program for nonlinear regression was used; it was based on the Levenberg-Marquardt algorithm (Fig. 3). This procedure is offered by *SigmaPlot 6.0*. The perfection of the fitting was tested by the sum of square deviations.

Table 2. The deconvolution parameters of the component bands of fluorescence spectra at 77 K of thylakoid membranes prepared from *Spirulina platensis* (Fig. 3).

Band	λ peak [nm]	Bandwith [nm]	Bandwith [cm^{-1}]	Area [r.u.]	Height [r.u.]
1	686	12	275	5.6	0.48
2	696	14	301	9.0	0.70
3	730	32	605	25.2	0.88
4	750	43	757	11.9	0.31

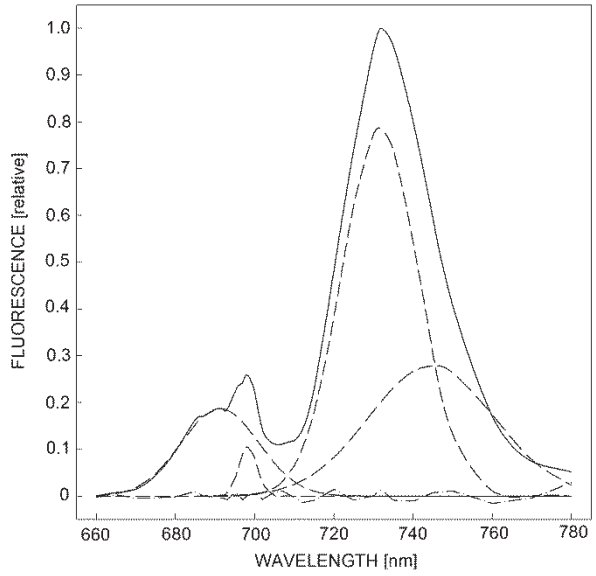

Fig. 3. Gaussian deconvolution of 77 K fluorescence emission spectrum recorded with thylakoids isolated from *Spirulina platensis* grown under irradiance fluorescent tubes *Lumilux 11* (*Osram*, Germany). The deconvolution analysis simulated four major bands traced by the *dash lines*.The parameters of these bands are given in Table 2. The bands with maxima at 686 and 696 nm are attributed to pigment proteins of PSII, the bands with maxima at 730 nm and 750 nm to PSI. The *dotted line* around the x-axis represents the residuals. For calculation of the PSII/PSI stoichiometry, only the band peaking at 730 nm was considered as representative of PSI.

Gel electrophoresis: SDS-PAGE was performed according to Laemmli (1970) at 5°C in the *Mini-PROTEAN II* (*BIO-RAD*, USA). The resolving polyacrylamide gel containing 8 M urea was prepared to form a linear concentration gradient of 10–20%. The concentration of the stacking gel was 5%. Sample amounts corresponding to 4 μg (Chl *a*) were applied to the gels. The proteins were stained with Coomassie Brilliant Blue G-250 or with silver according to Wray e*t al.* (1981).

Western blotting techniques: The electrophoretically separated proteins were transferred from an unfixed gel onto a nitrocellulose membrane (*NC, SCHLEICHER & SCHUELL,* USA) using the transfer apparatus *TRANS-BLOT (BIO-RAD*, USA) according to Towbin *et al.* (1979) at 60 V and 100 mA for 4 h. For immunoassay, the membrane was rinsed with PBS buffer (140 mM NaCl, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.2) and incubated for 2 h at room temperature with 5% skim milk powder (*OXOID*, Czech Republic) in PBS buffer. Strips of the nitrocellulose membrane were incubated for 90 min with rabbit antibodies serum, which was diluted 1:20, rinsed three times with PBS buffer containing 0.05% Tween 20 and finally incubated with peroxidase (*SwAM/Px, Serva*, USA, diluted 1:1,000), which was conjugated to swine antirabbit immunoglobulins (*SEVAC*, Czech Republic). The amidoblack-staining was carried out using 0.1% amidoblack (*Serva*, USA), 25% methanol, and 10% acetic acid.

Preparation of antibodies: Polyclonal antibodies against the D1, D2, and 33 kDa oxygen-evolving protein isolated from *S. platensis* were obtained by immunization of rabbits or mice. The proteins for the immunization were obtained from strips cut out from the preparative gels after denaturing electrophoresis. Gel strips were homogenized using laboratory homogenizator in buffer E (137 mM NaCl, 1.5 mM KH_2PO_4, 1.8 mM Na_2HPO_4, 2.7 mM KCl, pH 7.0) and the homogenate was used to start the immunization. A volume of the homogenate containing about 150 μg of protein was injected into subcutal space of rabbits and about 10 μg of protein were applied into the intraperitoneal space of mice. The booster injections containing 75 μg (rabbits) or 10 μg (mice) of protein were repeated every three weeks. The serum from the animals was collected by centrifugation (2,700 × *g*, 5 min, 4°C) after 9 weeks of immunization and stored frozen at –20°C.

Results and discussion

We made use of the experience acquired earlier by other authors and ourselves (Ritter *et al.* 1992, Murakami 1997) that the spectral quality of the growth irradiance modifies the molar ratio of the two photosystems in TM.

Therefore, the population of *S. platensis* was grown under three different light regimes specified in Materials and methods (Figs. 1,4). Stoichiometry of PSII and PSI complexes in the cells is usually assessed by two different quantities: (*1*) light-saturated rates of electron transport through PSI and PSII (PS-ETR) as measured with an

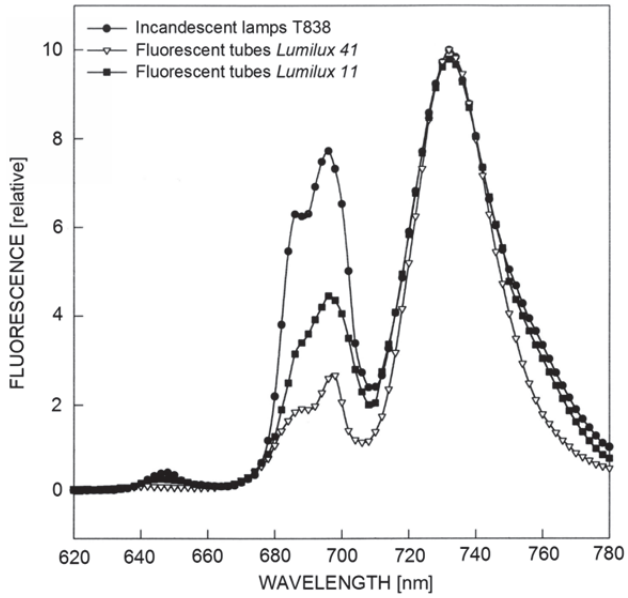

Fig. 4. 77 K fluorescence emission spectra of thylakoid membranes isolated from cells of *Spirulina platensis* grown under irradiance of different spectral composition. Excitation at λ = 436 nm. The curves are normalized to the maximum 735 nm. For characteristics of the radiation sources *see* Materials and methods and Fig. 1.

oxygen electrode; (*2*) the ratio of low temperature (77 K) fluorescence emission bands (LT–FER) estimated according to the areas under the major bands assumed to originate in the complexes of PSI and PSII. The method of deconvolution of our emission spectra is described and illustrated on the example in Materials and methods (Fig. 3). For quantitative calculations, we used the areas under the peaks obtained by deconvolution. The parameters of the curves obtained by deconvolution of the spectrum in Fig. 3 are given in Table 2. For quantitative characterization of PSI, we used only the area under the band peaking at 730 nm. The band with a maximum emission at 750 nm is of doubtful origin (Koehne and Trissl 1998, Schlodder *et al*. 2005). Table 3 shows the values of PSI and PSII ETR established in the cells grown under three different irradiation regimes as well as their ratios [$PSQ_{II/I\ (ETR)}$]. Table 4 indicates the same values measured with thylakoids isolated from the above cells. In addition, the ratios of areas under the fluorescence emission bands (Fig. 3) ascribed to PSII and PSI [$PSQ_{II/I\ (LT\text{-}FER)}$] are given.

Neither of the above quantities can be considered as an unequivocal measure of the molar quantities of two PS from which their ratio can be calculated. However, in the absence of other more reliable values, we used the PS-ETR and LT-FER as a reasonable guide in all

Table 3. Photochemical activity of *Spirulina platensis* cells grown in irradiance with various spectral composition. For the characteristics of the radiation sources, *see* Materials and methods and Fig. 1. PSII-ETR – electron transport through PSII measured as Hill reaction with electron acceptor 2,6-dichloro-*p*-benzoquinone, PSI-ETR – electron transport through PSI measured as Mehler reaction with methylviologen as electron acceptor. $PSQ_{II/I(ETR)}$ – quotient of the ETR through PSII (Hill reaction, HR) and through PSI (Mehler reaction, MR). The electron transport rates given in meq(e^-) mol^{-1}(Chl) s^{-1}. Mean ± SD (n = 5).

Radiation source	PSII-ETR	PSI-ETR	PSQII/I (ETR)
Incandescent lamps, T 838	1,512 ± 37.68	1,036 ± 41.44	1.457
Fluorescent tubes, Lumilux 11	1,364 ± 46.38	1,472 ± 23.25	0.921
Fluorescent tubes, Lumilux 41	840 ± 24.36	1,968 ± 78.46	0.426

considerations throughout this paper. Our attitude was based on the following facts: Murakami (1997) provided evidence that the ratio of molar quantities of the two PS in *Synechocystis* PCC 6714 assessed by redox difference spectroscopy (RO*Δ*A) corresponds reasonably with the ratio based on quantification of 77 K fluorescence emission. Even if the absolute values of the $PSQ_{II/I}$ based on LT-FER were lower than those obtained from RO*Δ*A, their relative changes under different conditions were proportional. The same correlation was supported by most of our measurements, in which the quotient based on ETR stands for Murakami's values based on RO*Δ*A. Several other authors consider the LT-FER values to be a reliable measure of PS molar quantities. (Cunningham *et al.* 1990, Salehian and Bruce 1992).

Our values of PSQ based on measurements of PS-ETR in the isolated thylakoids differed from those based on LT-FER (Table 4) more than the values found by Murakami with thylakoids of *Synechocystis*. In the latter case, the mean value of the ratio $PSQ_{RO\Delta A}/PSQ_{LT\text{-}FER}$ was 1.27, while in thylakoids of *Spirulina* we found the mean value of the $PSQ_{ETR}/PSQ_{LT\text{-}FER}$ ratio to be 1.51. In addition, our values for the PSQ ratios with thylakoids formed under lights of different spectral composition differed more (1.7–1.39) than did the corresponding figures of Murakami (1.31–1.23). The above differences were not surprising at all. Murakami's methods of quantifying the molar concentration of PS by RO*Δ*A depend on the quantum efficiency of PSI and PSII reaction centres. Our methods depended on the activity of the entire path of electron transport from the donors (H_2O in the case of PSII) to the acceptors that involved, in addition to the activity of the reaction centres, the limitations resulting from diffusion of donors and acceptors. In both cases, only the active centres were counted. On the other hand, LT-FER does not reflect the yield of the primary

Table 4. Photochemical and fluorescence characteristics of thylakoid membranes isolated from the cells grown under irradiance of various spectral composition. For the characteristics of the radiation sources, *see* Materials and Methods and Fig. 1. The specifications of the two sections of electron transport (through PSII and PSI) and their abbreviations are the same as in Table 3. LT-FER – ratio of low temperature (77 K) fluorescence emission maxima. $PSQ_{II/I\ (LT\text{-}FER)}$ − quotient of the LT-FER attributed to PSII and to PSI (estimated from areas under the LT-emission bands PSII and PSI, *see* text). PSQQ = $PSQ_{II/I\ (ETR)}$:$PSQ_{II/I\ (LT\text{-}FER)}$. Mean ± SD ($n$ = 5).

Source of irradiance	PSII- ETR	PSI-ETR	$PSQ_{II/I\ (ETR)}$	$PSQ_{II/I\ (LT\text{-}FER)}$	PSQQ
Incandescent lamps, T 838	1,360 ± 46.2	1,280 ± 39.7	1.06	0.58	1.70
Fluorescent tubes, Lumilux11	880 ± 30.8	1,320 ± 51.4	0.66	0.42	1.46
Fluorescent tubes, Lumilux 41	556 ± 21.7	1,696 ± 55.9	0.32	0.27	1.39

photochemical reaction and depends more on the structural intactness of the inner antennas, which control the maximum fluorescence yield. Furthermore, it is quite logical that the light-saturated PS-ETR measured by the conventional methods based on oxygen exchange with oxygen electrode are not related to the molar concentration of the photosynthetic unit by exactly the same proportionality factor if thylakoids of various organisms grown under various conditions (and therefore containing various structures) are examined. To quote only the most evident reason: the ETR values are referred to the molar concentration of Chl. The number of moles of Chl per 1 mole of photosynthetic unit may differ noticeably under the various conditions mentioned above. For similar reasons, the same molar quantity of PSII (or PSI) complexes originating from disparate conditions and having slightly different structures need not yield exactly the same rate of low temperature fluorescence. It is equally evident that one cannot assume the same fluorescence rates per mole of PSII and PSI complexes. It is rather surprising that a wide similarity was found in all these characteristics by Murakami (1997) and other authors quoted in their papers, as well as in our experiments. In view of the questions considered in this paper, it was decisive that the ranking of PSQ values according to two criteria were very much in parallel and the absolute values were not of basic importance. There was also a discrepancy between the quotients of PSII/PSI electron transport activities $PSQ_{II/I\ (ETR)}$ as measured in whole cells (Table 3) and in isolated thylakoids (Table 4). This difference might ensue from either of two causes: (*1*) PSII was more inactivated than PSI during the procedure of thylakoid isolation; (*2*) The ratio of the electron transport rates was actually different in the cells and in the isolated thylakoids due to some structural reasons. The ratio of the $PSQ_{II/I\ (ETR)}$ measured in thylakoids and in cells was very similar in all three cases documented in Table 3 and Table 4, varying only between 0.72 and 0.76. This suggests that the above alternative (*2*) was more probable.

Irrespective of the fact that absolute values of the PSQs were equivocal, it is obvious from the values in Tables 3 and 4 that the difference of PS stoichiometry conditioned by the spectral composition of the incident radiation exceeded a factor of three. In addition, the data conformed with the general rule: the highest value of the PSII:PSI ratio was obtained under the irradiance with prevalence of long wavelengths (in this case incandescent light). The lowest value was found in cells grown under fluorescent tubes emitting approximately equal flux densities of photons with wavelengths below and above 500 nm. Therefore we used cells grown under incandescent lamps for all isolation of PSII particles.

TM taken into all these comparisons were isolated by mechanical disruption of the cells with glass beads using a bead-beater at 2°C. This was at variance with the procedure used in our experiments with *S. elongatus.* This was the procedure routinely used earlier for thylakoid isolation from cyanobacterial cells, in particular from the thermophilic species of *Synechococcus* (Bowes *et al.* 1983, Schatz and Witt 1984) and *Mastigocladus laminosus* (Bohler and Binder 1980). In this procedure, the first obligate step was loosening of the rigid peptidoglycan layer by the enzymatic action of lysozyme. When various strains of *Synechocystis* became the most popular organisms in this field, it was found that lysozyme treatment fails to bring the expected effect and that bare mechanical disruption of the cells is more efficient (Burnap *et al.* 1989, Rögner *et al.* 1990, Tang and Diner 1994, Shen and Vermaas 1994, Barbato *et al.* 1995). We found that *Spirulina* behaved similarly to *Synechocystis*, *i.e.* it did not also yield to the action of lysozyme in a desirable way.

Therefore we tried three other approaches. The first was the abovementioned mechanical disintegration by vibrating the cells with small glass beads, the second was the disruption of the cells by the pressure jump in the French press. Our third trial issued from the assumption that the external fibrous sheath, consisting often of lipopolysaccharides, might preclude in *Spirulina* either the action of lysozyme or the rupture of the cells with the murein layer destroyed. We tried, therefore, incubation of the cells with various enzymes hydrolyzing cell wall polysaccharides.

The results of the comparative experiment were summarized in Table 5. The quality of the thylakoids isolated was estimated by the rates of electron transport through both PS. The harsh mechanical disintegration yielded noticeably better results than the milder procedures involving pressure jump.

Concerning the cellulytic enzymes, the whole effort

Table 5. Oxygen exchange of thylakoid membranes isolated using glass beads, French press, and enzymes. PSII-ETR – electron transport through PSII measured as Hill reaction with electron acceptor 2,6-dichloro-*p*-benzoquinone, PSI-ETR – electron transport through PSI measured as Mehler reaction with methylviologen as electron acceptor. $PSQ_{II/I(ETR)}$ − quotient of the ETR through PSII (Hill reaction, HR) and through PSI (Mehler reaction, MR). The electron transport rates given in mequ(e^-) mol^{-1}(Chl) s^{-1}. Mean ± SD (n = 5).

Isolation method	PSII-ETR	PSI-ETR	$PSQ_{II/I\ (ETR)}$
Mechanical disruption with glass beads	1,296 ± 19.44	1,140 ± 30.78	1.13
Disintegration with French press	968 ± 31.82	940 ± 30.08	1.02
Lysozyme + hemicellulase from *Aspergilus niger*	532 ± 12.24	576 ± 23.04	0.92
Lysozyme + pectinase from *Aspergilus niger*	652 ± 20.17	772 ± 27.02	0.86

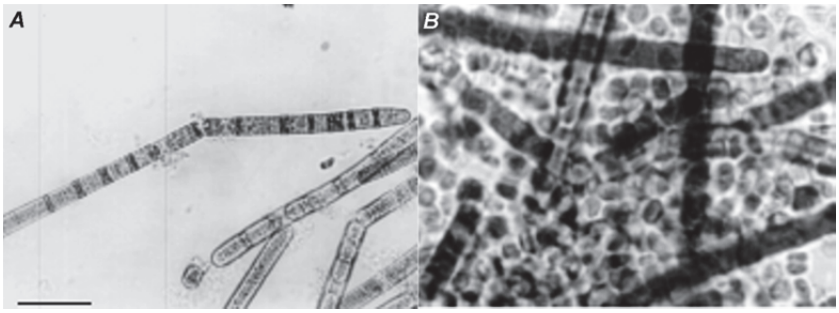

Fig. 5. Microphotographs showing the effects of the enzymes on the cell walls of *Spirulina*. *A*: after 60-min incubation with lysozyme (*Serva* 28262, Germany). *B*: after 90-min incubation with a mixture of lysozyme and pectinase (*Aspergillus niger* R-5146, *Sigma,* USA). A significant proportion of cells released protoplasts. Bars = 25 μm.

Table 6. Photochemical activities of PSII crude particles prepared by detergent extractions performed under conditions described in Table 1. The specifications of electron transport through PSII and PSI and their abbreviations are the same as in Table 3. Hill reaction (HR) was measured with electron acceptors 2,6-dichloro-*p*-benzoquinone (DCBQ), 2,6-dimethyl-*p*-benzoquinone (DMBQ), and *p*-benzoquinone (BQ). PSI-ETR, PSII-ETR – electron transport through PSI, PSII; respectively. Mean ± SD (n = 5). SB12, DM, OG, HTG, OTG, DGP, LDAO – *see Abbreviations*.

PSII particles	PSII-ETR, HR			PSI-ETR	Extraction yield [%]
	DCBQ	DMBQ	BQ		
DM	1,556 ± 54	1,504 ± 75	1,472 ± 59	228± 9	77.5
OTG	1,248 ± 43	1,024 ± 41	864 ± 26	300 ±9	70.8
OG	772 ± 31	648 ± 13	588 ± 24	236 ± 12	79.6
SB12	808 ± 43	720 ± 36	488 ± 24	304 ± 11	60.9
HTG	200 ± 11	128 ± 4	180 ± 5	148 ± 4	11.3
LDAO	268 ± 5	260 ± 10	224 ± 4	340 ± 14	56.4
DGP	120 ± 6	120 ± 4	108 ± 3	248 ± 5	19.6
Triton X-100	308 ± 9	300 ± 9	268 ± 8	864 ± 26	57.6

was a failure. Only two of eight tested enzymes showed any noticeable effect, but none of them was practically useful. These were the pectinase from *Aspergillus niger* (*R–5146*, *Sigma,* USA) and hemicellulase from *A. niger* (*H–8125*, *Sigma, USA*). Micrographs of the cells upon treatment with these enzymes are shown in Fig. 5. The prolapsed flocks of protoplasm in Fig. 5*A* might indicate that lysozyme penetrated to the murein layer. Thus holes in the lipopolysaccharide layer opened by the hemicellulase released protoplasts of the cells (Fig. 5*B*).

Since the vibrating mill with glass beads proved to be the most efficient, we used it routinely for isolation of TM. The thylakoids were then incubated with various detergents under conditions described in the Materials and methods (Table 1). We used a set of detergents that comprised nearly all of those used in our earlier experiments with *Synechococcus elongatus*. This made possible to compare the efficiency of the detergents in selective extraction of PS particles from membranes of two distinctly different cyanobacteria, *i.e.* the thermophilic *Synechococcus* and the mesophilic *Spirulina*.

Electron transport rates (ETR) through PSII and PSI characterizing the crude particle suspensions obtained by various detergents are summarized in Table 6. It was evident that the detergents could be separated in two groups. In the first group, we could rank the extracts with relatively high rates of PSII electron transport measured as oxygen production in the Hill reaction with 2,6-dichloro-*p*-benzoquinone (DCBQ) as the electron acceptor: values between 800 and 1,500 meq(e^-) mol^{-1}(Chl) s^{-1}. The second one, accommodated extracts with markedly lower PSII activities in the range of 120 to 310 meq(e^-) mol^{-1}(Chl) s^{-1}.

We concluded further that the first group was

characterized by higher total yields of PS extraction judged from the fraction of total Chl in TM recovered in the final supernatant containing the particles. This was 60–80% in the 1st group and 10–60% in the 2nd one. Surprisingly, the PSI activity of most extracts referred to unit of Chl varied in a comparatively narrow range, *i.e.* from 145 to 350 meq(e^-) mol^{-1}(Chl) s^{-1} with the exception of Triton X-100 extracts, in which evidently the PSI particles strongly predominated.

The results were ambiguous, since the rate of PSI or PSII activity referred to unit of Chl content was a product of the molar concentration of the respective (PSI or PSII) particles and their specific (molar) activity divided by the total Chl content of the extract. Thus, a lower activity might be caused equally by a lower number of fully active particles as by a higher number of particles, some of which were inactive. This uncertainty is particularly inconvenient if the aim is to screen for a homogeneous suspension of functionally and structurally intact complexes.

In the extracts of the first group, in particular in the DM-PSII particles with the highest PSII activity, we might assume with a high probability that the measured activity per unit of Chl reflected largely the concentration of the active centres. Active reaction centre (RC) of PSI and PSII containing only the "inner antenna of RC" has approximately equal amounts of Chl molecules per RC complex. Cyanobacterial PSI RC contains 6 RC-Chl, cyanobacterial PSII RC contains 4–6 RC-Chl (Xiong *et al.* 1996). In that case, the fully active particles of PSII and PSI display comparable values of activity referred to unit of Chl amount bound in the respective PS particles. The low activities of extracts in the 2nd group indicated, most probably, a considerable proportion of inactive particles of both PS.

Comparison of oxygen production rates with different substituted benzoquinones as electron acceptors provides valuable information on the state of the acceptor side of PSII. Takana-Kitatani *et al*. (1990) proposed that when the rates of Hill reaction with BQ, DMBQ, and DCBQ are not significantly different, the acceptor side of the PSII complexes examined is well preserved. Among the extracts of the first group (DM, OTG, OG, SB12 – *see Abbreviations*), only the DM-PSII particles met this criterion (Table 6). In particles extracted by three other detergents (HTG, LDAO, DGP– *see Abbreviations*), the oxygen evolution rates supported by different acceptors often varied. In addition, these particles displayed lower values of PSII activity in general, which pointed to damage in a different part of the complex, most probably at the donor side of PSII RC. The oxygen-evolving activity of particles in the 2nd group of Table 6 was strongly reduced without any marked discrimination between different electron acceptors. Here again, a damage of PSII RC or of the oxygen-evolving complex was the most probable.

Table 7 shows the $PSQ_{II/I\ (ETR)}$ of the individual extracts calculated from the data in Table 6. It shows further the values of $PSQ_{II/I\ (LT\text{-}FER)}$ determined by the same procedure as used for assessment of corresponding values for TM (*see* Table 6). For extracts in the 1st group, the calculated ratios of the PSQ values were very similar to ratios found in the thylakoids. This was a convenient confirmation of our assumption that the corresponding difference between PSQ_{ETR} and $PSQ_{LT\text{-}FER}$ reflects the true functional properties of PS in the thylakoids of *S. platensis*. The agreement of the values of PSQQ with active particles of the first group provided also a circumstantial evidence that the PSQs indicated by both methods came near to the molar ratio of PS. The fact that PSQs values for the inactive extracts of the second group differ might be related in some way to the fact that these extracts contained a large proportion of damaged and inactive particles.

If we took the values of $PSQ_{II/I\ (ETR)}$ for basis, then the selectivity of all the detergent extractions for PSII complexes was low, particularly, as compared to the action of detergents on the thylakoids of *S. elongatus*. Since the value of the $PSQ_{II/I\ (ETR)}$ in the thylakoids, to which the detergents were applied, was 1.06 (Table 4), the value for the most efficient detergent, *i.e.* DM, exhibited a six times higher preference for PSII in

Table 7. The purity of PSII particles the activity of which is given in Table 6. The specifications of electron transfer and low temperature (77 K) fluorescence emission ratio measurements through PSII, PSI are the same as in Table 3 and Table 4. SB12, DM, OG, HTG, OTG, DGP, LDAO – *see Abbreviations*.

PSII particles	$PSQ_{II/I(ETR)}$	$PSQ_{II/I(LT\text{-}FER)}$	PSQQ
DM	6.8	4.6	1.47
OTG	4.16	2.9	1.43
OG	3.27	2.16	1.51
SB12	2.65	1.8	1.47
HTG	1.4	1.33	1.05
LDAO	0.8	-	-
DGP	0.5	0.75	0.66
Triton X-100	0.4	-	-

Table 8. Rate of electron transport (ETR) through photosystem II (PSII) in DM-PSII (*see* Abbreviations) particles fractionated on sucrose-density gradient. The specifications of electron transfer measurements through PSII and PSI are the same as in Table 3. Mean ± SD (n = 5).

Zone No.	Sucrose [%]	Chlorophyll [% of total]	PSII-ETR	PSI-ETR
1	25	6 ± 0.19	0	0
2	30	16 ± 0.77	88	0
3	40	23 ± 0.54	668	156
4	43	42 ± 2.27	1,684	40
5	45	13 ± 0.63	80	128
DM-PSII		100	1,556	228

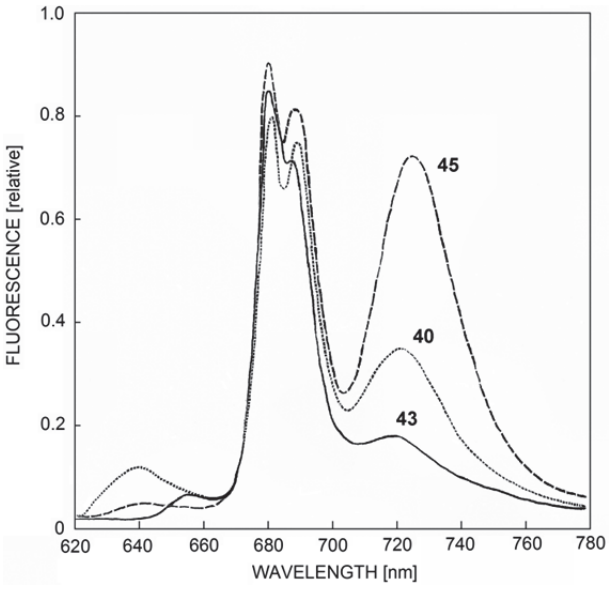

Fig. 6. 77 K fluorescence emission spectra of DM-PSII particles obtained from zones of sucrose-density gradient centrifugation (160,000 × *g*, 16 h, 4°C) in the sucrose concentration gradient (20–40%). The numbers labelling the curves indicate the concentration of sucrose in the respective zone.

comparison to PSI. Two of the detergents in the 2nd group showed a slight preference for PSI and Triton X-100 displayed a noticeably higher affinity to PSI than to PSII. The preferential extraction of PSI particles from cyanobacteria by Triton X-100 was presented also by Golbeck *et al.* (1988), Komenda *et al.* (1989), and Šetlíková *et al.* (1999). The DM extract provides not only a high yield of PSII particles, but the latter seem also to be the least damaged functionally. Apart from the generally high rates of oxygen evolution, these rates were also very similar with all three quinone acceptors used. According to the general consent commented in more detail in Šetlíková *et al.* 1999, this indicated that the structure of the PSII complex at the acceptor side (up to the Q_B pocket) was undisturbed and that the transfer of electrons from Q_A^- to the plastoquinone molecules in the Q_B pocket worked well.

DM extract was subjected to a further purification step through centrifugation on the sucrose density gradient. Individual zones from the sucrose density gradient were documented in more detail by the ETR data (Table 8) and by the spectral characteristics (Fig. 6). Zones 1 and 2 contained mainly free carotenoids, phycobilins, and Chls. The zone 3 contained a mixture of PSI and PSII (probably monomers). The zone 5 were mainly PSI trimmers and a rest of nonsolubilized of PSII. In the main zone 4, encompassing about 40% of the total Chl loaded on the gradient and containing PSII, probably dimers, PSII activity strongly predominated. This was an important finding since it demonstrated that in spite of a negligible preference for PSII in the extraction procedure, the particles of both PS were present in the extract separately and they could be dissociated by an appropriate procedure. The relatively poor enhancement of the PSII:PSI ratio in the crude extract could also result from the association of both complexes in the same membrane fragments. Our conclusions from data obtained by means of sucrose density gradient were supported by Zheleva *et al.* (1998).

Since the yield of total Chl recovered in the supernatant of DM extract was about 75% of TM Chl concentration, the final yield of the purified PSII particles recovered in the fraction 4 of the gradient was 0.75 × 0.4 = 0.3, *i.e.* 30% of the Chl taken into the extraction. This was a reasonable figure compared with results of other authors.

The polypeptide composition of the fraction 4 from the sucrose gradient was compared with that of the crude (*lane 1*) and the purified DM-extract (*lane 5*) in Fig. 7. The marked reduction of the polypeptides derived from PSI was mainly apparent in the area of the trimers of M_r around 210 kDa. We assumed that the marked band with mobility corresponding to relative molecular mass (M_r) around 85 kDa could be ascribed to the large linker polypeptide (anchor protein) as described by Gantt *et al.* (1988) and Egashira *et al.* (1995) in thermophilic cyanobacterium, *Synechococcus vulcanus.* Similarly, the band at M_r 66 could be the most probably identified as the D1-D2 dimer described in more detail by Barber *et al.* (1987) and the band at M_r above 40 as the cross-linked adduct of D1 protein and α-subunits of cytochrome b_{559} characterized by Barbato *et al.* (1992). In both these bands, we could demonstrate the presence of the epitope belonging to D1, to which we raised antibodies (Fig. 7, *lane 4*). This was in contrast to the antibodies recognizing the C-terminal parts of the D1 and the D2 polypeptides

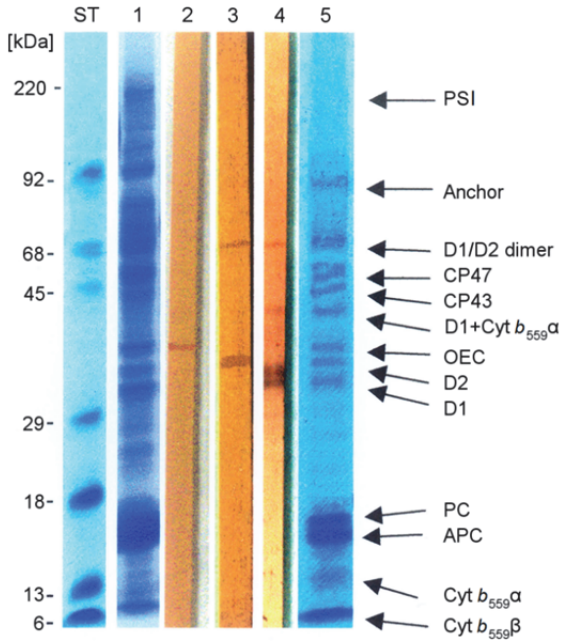

Fig. 7. SDS-PAGE of proteins constituting the crude (*lane 1*) and the purified DM-PSII particles (*lane 5*). Western blots of the latter with polyclonal antibodies prepared as described in Materials and methods, are shown in *lanes 2–4*. *Lane 2* shows the position of the PsbO protein. *Lane 3* shows that the anti-D2 labels the D2 monomer as well as the D1, D2 heterodimer. The same holds for the anti-D1, which, in addition, indicates the presence of the D1-Cyt b_{559} α adduct (*lane 4*). Line ST: migration distance of protein standards with molecular masses indicated on the left. APC – allophycocyanine; PC – phycocyanine.

(Nixon *et al.* 1995), which did not show any affinity to our putative D1-D2 dimer. This difference in the immunospecific reaction of both types of antibodies was not limited to the polypeptide bands derived from the DM-PSII particles. We observed it also in the blots of polypeptides from other electrophoretograms of *Spirulina* thylakoid proteins. We concluded therefore that different epitopes were involved and that the C-terminal part, for which Nixon's antibodies were specific, was not accessible in the structure of the dimer present in the SDS polyacrylamide gels.

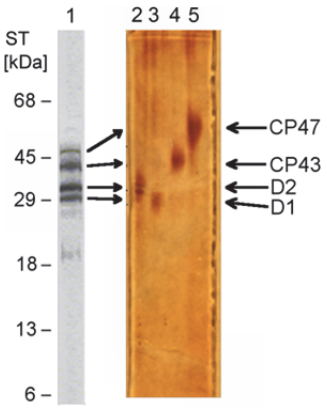

Fig. 8. SDS-PAGE of the constituents of DM PSII-core complex (*lane 1*). *Lanes 2, 3, 4 and 5* show re-electrophoresis of polypeptides from the individual bands in the gel of *lane 1*. Column ST indicates the position of molecular mass standards. Gels from the consecutive electrophoresis were stained with silver.

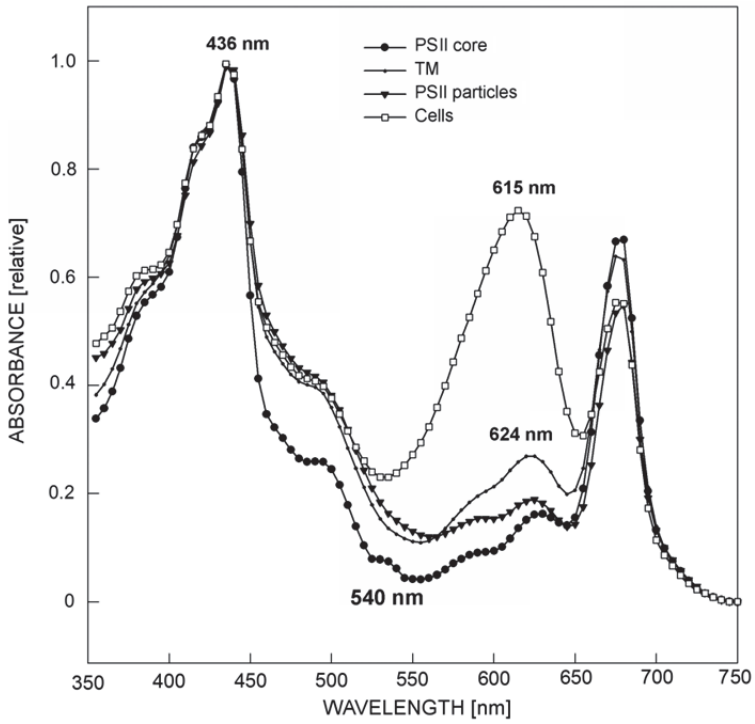

Fig. 9. Absorption spectra of *Spirulina platensis* cells, thylakoids, DM-PSII crude and DM-PSII core complexes. Spectra are normalized to the maximum absorption at 436 nm. The position of the maximum absorption in the red band is given for individual curves between 677 and 685 nm. The peaks between 600 and 650 nm belong to phycobiliproteins, the shoulder at 540 nm indicates presence of pheophytin *a*. TM – thylakoid membranes.

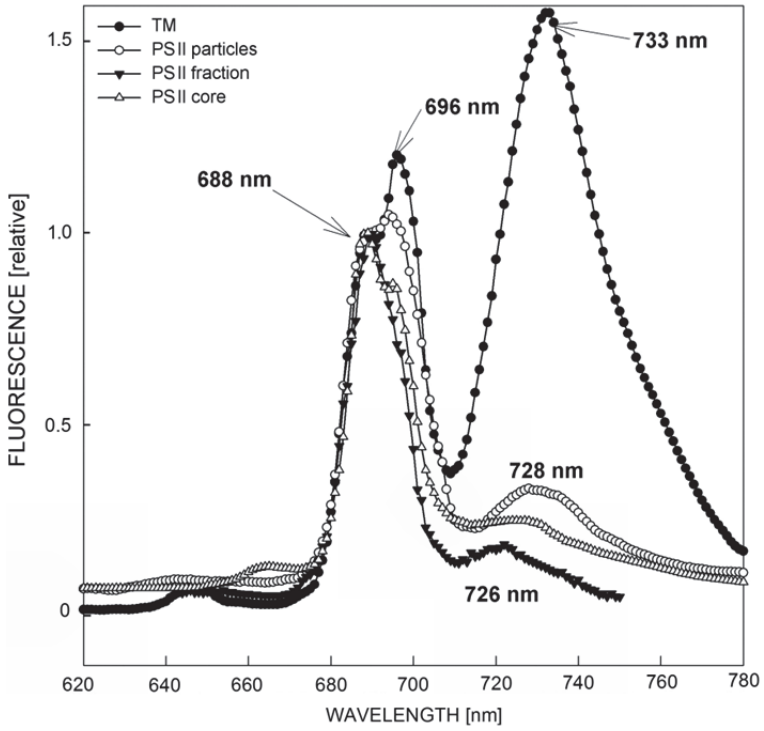

Fig. 10. 77 K fluorescence emission spectra of thylakoid membranes (TM), crude (DM-PSII particles), purified DM-PSII particles on the sucrose density gradient (PSII fraction) and the PSII-core complex. Excitation wavelength λ = 436nm. The spectra are normalized to the emission at 688 nm.

Since the DM extract had the highest relative content of PSII complexes, we used it for the preparation of the core complex particles. This was achieved with immobilized metal affinity chromatography (IMAC) with Cu^{2+} loaded Sepharose, which was proved earlier as appropriate to this purpose (Šetlíková *et al.* 1995). The modification of the procedure applied here was described in the Materials and methods.

The polyacrylamide gel electrophoretogram (Fig. 8, *lane 1*) showed the principal polypeptides constituting the core complex: the components of the reaction centre D1, D2 as well as the inner antenna polypeptides, CP43, and CP47. Contrary to Zheleva *et al*. (1998), who prepared the subcomplex core containing D1, D2, and CP47, we used the Cu^{2+}-IMAC method. With this method, the cytochrome b_{559} freqently remains bound on the column, from which it is finally set free using more drastic eluents, *e.g*. Triton X-100 (Šetlíková *et al*. 1995, Fig. 4, *lane 6*).

Komenda *et al*. (2012) also prepared a core complex of the composition of D1, D2, CP43, and CP47 using Ni^{2+}-IMAC. This method is particularly suitable for preparation of the core complex containing D1, D2, CP43, and CP47.

The 33 kDa protein of oxygen-evolving complex (OEC) is very unstable, being frequently set free from the column, when phycobiliproteins are being removed from the bound PSII-DM complex. This was the reason, why we used SDS-PAGE bands of TM to prepare antibodies.

The slightly weaker band of CP47 (Fig. 8, *lane 1*) suggested that a part of the complexes was deprived of this inner antenna component. The purity of the bands and the identity of the components were confirmed by consecutive electrophoresis of the bands with silver staining of the gels (Fig. 8, *lanes 2,3,4,5*).

The absorption spectrum of PSII core complex (Fig. 9) showed typical features: the red maximum at 678 nm, and a complex shoulder on the long wave arm of the Soret band, which is a contribution from carotenoids around 480 nm and that of pheophytin at 540 nm. Fig. 10 shows the 77 K fluorescence emission spectra of the individual steps in preparation of the PSII core complex (TM, PSII crude particles, PSII sucrose fraction, and PSII core complex). We assumed that the fluorescence emission with maximum at 686-688 nm originated in CP 43 and that the peak around 696 nm came from the Chl molecules in CP47 (Nakatani 1984, Shen and Vermaas 1994). Consequently, we ascribed the lowering of the emission maximum and its blue shift from 696–694 nm (Fig. 10) to a partial loss of the CP 47 pigment-protein documented by the SDS-PAGE (Fig. 8, *lane 1*). Furthermore, we assumed that the low fluorescence band with maximum at 726–730 nm emitted from the core complex did not originate in the PSI Chl-protein, but it represented a satellite emission from the PSII components (van Dorssen *et al.* 1987). This view was supported by the fact that no trace of a PSI characteristic band could be seen in the SDS-PAGE (Fig. 8).

References

Arnon, D.I.: Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. – Plant Physiol. **24**: 1-15, 1949.

Barbato, R., de Laureto, P.P., Rigoni, F. *et al*.: Pigment-protein complexes from the photosynthetic membrane of the cyanobacterium *Synechocystis* sp. PCC 6803. – Eur. J. Biochem. **234**: 459-465, 1995.

Barbato, R., Friso, G., Rigoni, F. *et al*.: Characterization of a 41 kDa photoinhibition adduct in isolated photosystem II reaction centres. – FEBS Lett. **309**: 165-169, 1992.

Barbato, R., Mulo, P., Bergo, E. *et al*.: Substantial deletions in the DE loop of the photosystem II D1 protein do not prevent its turnover or cross-linking with the *α*-subunits of cytochrome b_{559}. A study using *Synechocystis* sp. PCC6803 mutants. – J. Plant Physiol. **154**: 591-596, 1999.

Barber, J., Chapman, D.J., Telfer, A.: Characterization of a PSII reaction centre isolated from the chloroplasts of *Pisum sativum*. – FEBS Lett. **220**: 67-73, 1987.

Bartoš, J., Berková, E., Šetlík, I.: A versatile chamber for gas exchange measurements in suspensions of algae and chloroplasts. – Photosynthetica **9**: 395-406, 1975.

Bohler, M.-C., Binder, A.: Photosynthetic activities of a membrane preparation of the thermophilic cyanobacterium *Mastigocladus laminosus*. – Arch. Microbiol. **124**: 155-160, 1980.

Bowes, J.M., Stewart, A.C., Bendall, D.S.: Purification of photosystem II particles from *Phormidium laminosum* using the detergent dodecyl-β-D-maltoside. Properties of the purified complex. – Biochim. Biophys. Acta **725**: 210-219, 1983.

Bricker, T.M., Morvant, J., Masri, N., Sutton, H.M., Frankel, L.K.: Isolation of a highly active Photosystem II preparation from *Synechocystis* 6803 using a histidine-tagged mutant of CP 47. – Biochim. Biophys. Acta **1409**: 50-57, 1998.

Burnap, R., Koike, H., Sotiropoulou, G. *et al*.: Oxygen evolving membranes and particles from the transformable cyanobacterium *Synechocystis* sp. PCC6803. – Photosynth. Res. **22**: 123-130, 1989.

Chua, N.-H.: Photooxidation of 3,3´-diaminobenzidine by blue-green algae and *Chlamydomonas reinhardii*. – Biochim. Biophys. Acta **267**: 179-189, 1972.

Cunningham, F.X.,Jr., Dennenberg, R.J., Jursinic, P.A., Gannt, E.: Growth under red light enhances Photosystem II relative to Photosystem I and phycobilisomes in red alga *Phorphyridium cruentum*. – Plant Physiol. **93**: 888-895, 1990.

Debus, R.J., Barry, B.A., Babcock, G.T., McIntosh, L.: Site-directed mutagenesis identifies a tyrosine radical involved in the photosynthetic oxygen-evolving system. – P. Natl. Acad. Sci. USA **85**: 427-430, 1988.

Doucha, J., Kubín, Š.: Measurement of *in vivo* absorption spectra of microscopic algae using bleached cells as a reference sample. – Arch. Hydrobiol. **49**: 199-213, 1976.

Egashira, H., Kashino, Y., Koike, H., Satoh, K.: Isolation of photosystem II core complexes from a cyanobacterium without using column chromatography. – In: Mathis, P. (ed.): Photosynthesis: from Light to Biosphere. Vol.I. Pp. 627-630. Kluwer Acad. Publishers, Dordrecht – Boston – London 1995.

Ferreira, K.N., Iverson, T.M., Maghlaoui, K. *et al*.: Architecture of the photosynthetic oxygen-evolving center. – Science **303**: 1831-1833, 2004.

Fork, D.C., Murata, N., Sato, N.: Effect of growth temperature on the lipid and fatty acid composition and the dependence on temperature of light-induced redox reactions of cytochrome *f* and of light energy redistribution in the thermophilic blue-green alga *Synechococcus lividus*. – Plant Physiol. **63**: 524-530, 1979.

Gantt, E., Cunningham, F.X., Lipschultz, C.A., Mimuro, M.: N-terminus conservation in the terminal pigment of phycobilisomes from a prokaryotic and eukaryotic alga. – Plant Physiol. **86**: 996-998, 1988.

Golbeck, J.H., Parrett, K.G., Mehari, T., Jones, K.L., Brand, J.J.: Isolation of intact photosystem I reaction center core containing P700 and iron-sulfur center F_X. – FEBS Lett. **228**: 268-272, 1988.

Guskov, A., Kern, J., Gabdulkhakov, A. *et al*.: Cyanobacterial photosysten II at 2.9 Å resolution and the role of quinines, lipids, channels and chloride. – Nat. Struct. Mol. Biol. **16**: 334-342, 2009.

Hillmann, B., Schlodder, E.: Electron transfer reactions in Photosystem II core complexes from *Synechococcus* at low temperature — difference spectrum of $P680^+ Q_A^-$/P680 Q_A at 77 K. – Biochim. Biophys. Acta **1232**: 76-88, 1995.

Kamiya, N., Shen, J.R.: Crystal structure of oxygen-evolving photosystem II from *Thermosynechococcus vulkanus* at 3.7-Å resolution. – P. Natl. Acad. Sci. USA **100**: 98-103, 2003.

Kern, J., Loll, B., Lüneberg, C. *et al*.: Purification, characterization and crystalization of photosystem II from *Thermosynechococcus elongatus* cultivated in a new type of photobioreactor. – Biochim. Biophys. Acta **1706**: 147-157, 2005.

Koehne, B., Trissl, H.-W.: The cyanobacterium *Spirulina platensis* contain a long-vawelength absorbing pigment C_{738} (F_{760}, 77K) at room temperature. – Biochemistry **37**: 5494-5500, 1998.

Koike, H., Kashino,Y., Satoh, K.: [Interaction of halogenated benzoquinones with the non-heme iron (Q_{400}) in photosystem II]. – Z. Naturforsch. **48**: 168-173, 1992. [In German]

Komenda, J., Hladik, J., Sofrova, D.: On the multiple forms of cyanobacterial PS1 complex. – J. Photochem. Photobiol. B. Biology **3**: 575-592, 1989.

Komenda, J., Knoppová, J., Kopečná, J. *et al*.: Psb27 assembly factor binds to the CP43 complex of photosystem II in the cyanobacterium *Synechocystis* sp. PCC 6803. – Plant Physiol. **158**: 476-486, 2012.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of hot bacteriophage T4. – Nature **227**: 680-685, 1970.

Müh, F., Renger, T. Zouni, A.: Crystal structure of cyanobacterial photosystem II at 3.0 Å resolution: A closer look at the antenna system and the small membrane-intrinsic subunits. – Plant Physiol. Biochem. **46**: 238-264, 2008.

Murakami, A.: Quantitative analysis of 77K fluorescence emission spectra in Synechocystis sp. PCC 6714 and Chlamydomonas reinhardtii with variable PSI/PSII stoichiometries. – Photosynth. Res. **53**: 141-148, 1997.

Murata, N., Wada, H., Gombos, Z.: Modes of fatty-acid desaturation in cyanobactaria. – Plant Cell Physiol. **33**: 933-941, 1992.

Nakatani, H.Y., Ke, B., Dolan, E., Arntzen, C.J.: Identity of the Photosystem II reaction center polypeptide. – Biochim. Biophys. Acta **765**: 347-353, 1984.

Nixon, P.J., Komenda, J., Barber, J. *et al*.: Deletion of the PEST-like region of Photosystem II modifies the Q_B-binding pocket but does not prevent rapid turnover of D1. – J. Biol. Chem. **270**: 14919-14927, 1995.

Noren, G.H., Boerner, R.J., Barry, B.A.: EPR characterization

of an oxygen-evolving photosystem II preparation from the transformable cyanobacterium Synechocystis 6803. – Biochemistry **30**: 3943-3950, 1991.

Ritter, R., Komenda, J., Šetlikova, E., Šetlík, I., Welte, W.: Immobilized metal affinity chromatography for the separation of photosystems I and II from the thermophilic cyanobacterium *Synechococcus elongatus.* – J. Chromatogr. **625**: 21-31, 1992.

Rögner, M., Nixon, P.J., Diner, B.A.: Purification and characterization of photosystem I and photosystem II core complexes from wild-type and phycocyanin-deficient strains of the cyanobacterium *Synechocystis* PCC 6803. – J. Biol. Chem. **265**: 6189-6196, 1990.

Salehian, O., Bruce, D.: Distribution of excitation energy in photosynthesis: Quantification of fluorescence yields from intact cyanobacteria. – J. Lumin. **51**: 91-98, 1992.

Satoh, K., Oh-hashi, M., Kashino, Y., Koike, H.: Mechanism of electron flow through the Q_B site in photosystem II. 1. Kinetics of the reduction of electron acceptors at the Q_B and plastoquinone sites in photosystem II particles from the cyanobacterium *Synechococcus vulkanus*. – Plant Cell Physiol. **36**: 597-605, 1995.

Schatz, G.H., Witt, H.T.: Extraction and characterization of oxygen-evolving Photosystem II complexes from a thermophilic cyanobacterium *Synechococcus* spec. – Photobiochem. Photobiophys. **7**: 1-14, 1984.

Schlodder, E., Çetin, M., Byrdin, M. *et al*.: $P700^+$- and $^3P700$-induced quenching of the fluorescence at 760 nm in trimeric Photosystem I complexes from the cyanobacterium *Arthrospira platensis*. – Biochim. Biophys. Acta **1706**: 53-67, 2005.

Schlősser, U.G.: [Origin and function of the culture collection. – Ber. Deut. Bot. Ges. **95**: 181-276, 1982.] [In German]

Šetlíková, E., Ritter, S., Hienerwadel, R. *et al*.: Purification of a Photosystem II reaction center from the thermophilic cyanobacterium using immobilized metal affinity chromatography. – Photosynth. Res. **43**: 201-211, 1995.

Šetlíková, E., Sofrová, D., Prášil, O. *et al*.: Integrity and activity of photosystem II complexes isolated from the thermophilic cyanobacterium *Synechococcus elongatus* using various detergents. – Photosynthetica **37**: 183-200, 1999.

Shen, G., Vermaas, W.F.J.: Chlorophyll in a *Synechocystis* sp. PCC 6803 mutant without photosystem I and photosystem II core complex. Evidence for peripheral antenna chlorophylls in cyanobacteria. – J. Biol. Chem. **269**: 13904-13910, 1994.

Stewart, A.C., Bendall, D.S.: Preparation of an active, oxygen-evolving photosystem 2 particle from a blue-green alga. – FEBS Lett. **107**: 308-312, 1979.

Takana-Kitatani, Y., Satoh, K., Katoh, S.: Interaction of benzoquinones with Q_A^- and Q_B^- in oxygen-evolving photosystem II particles from the thermophylic cyanobacterium *Synechococcus elongatus*. – Plant Cell Physiol. **31**: 1039-1047, 1990.

Tang, X.-S., Diner, B.A.: Biochemical and spectroscopic characterization of a new oxygen evolving photosystem II core complex from the cyanobacterium *Synechocystis* PCC 6803. – Biochemistry **33**: 4594-4603, 1994.

Towbin, H., Staehlin, T., Gordon, J.: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. – P. Natl. Acad. Sci. USA **76**: 4350-4354, 1979.

Umena, Y., Kawakami, K., Shen, J-R., Kamiya, N.: Crystal structure of oxygen-evolving photosystem II at a resolution 1.9 Å. – Nature **473**: 55-60, 2011.

van Dorssen, R.J., Breton, J., Plijter, J.J. *et al*.: Spectroscopic properties of the reaction center and of the 47 kDa chlorophyll protein of Photosystem II. – Biochim. Biophys. Acta **893**: 267-274, 1987.

Vermaas, W.F.J., Williams, J.G.K., Arntzen, C.J.: Side-directed mutations of two histidine residues in the D2 protein inactivate and destabilize photosystem II in the cyanobacterium *Synechocystis* 6803. – Z. Naturforsch. **42c**: 762-768, 1987.

Warburg, O., Lűttgens, W.: Experiments on assimilation of carbon dioxide.] – Naturwissenschaften **32**: 161-167, 1944. [In German]

Wada, H., Murata, N.: Temperature-induced changes in the fatty acid composition of the cyanobacterium PCC6803. – Plant Physiol. **92**: 1062-1069, 1990.

Wray, W., Boulikas, T., Wray, V., Hancock, R.: Silver staining of proteins in polyacrylamide gels. – Anal. Biochem. **118**: 179-203, 1981.

Xiong, J., Govind, J., Subramanian, S.: Modeling of the D1/D2 proteins and cofactors of the II reaction center: Implication for herbicide and bicarbonate binding. – Protein Science **5**: 2054-2073, 1996.

Zheleva, D., Sharma, J., Panico, M. *et al*.: Isolation and characterization of monomeric and dimeric CP47-reaction center photosystem II complexes. – J. Biol. Chem. **273:** 16122-16127, 1998.

Zouni, A., Lüneberg, C., Fromme, P. *et al*.: Characterization of single crystals of Photosystem II from the thermophilic cyanobacterium *Synechococcus elongatus*.-In: Garab, G.. (ed.): Photosynthesis: Mechanisms and Effects. Vol. II. Pp.925-928. Kluwer Acad. Publishers, Dordrecht – Boston – London 1998.

Zouni, A., Witt, H.T., Kern, J. *et al*.: Crystal structure of photosystem II from *Synechococcus elongatus* at 3.8 Å resolution. – Nature **409**: 739-743, 2001.