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ISOLATION OF A TENFOLD HIS-TAGGED D1 PROTEIN FROM CHLAMYDOMONAS REINHARDTII USING NI-NTA AGAROSE

To make the D1 protein available for restriction studies and modification analysis, a pure D1-preparation which realizes the accumulation of the D1 protein by a fast and reproducible method, is necessary. The current work presents such a method. Inside the D1 protein of the chlorophycee Chlamydomonas reinhardtii a tenfold His-tag was integrated into the d-e loop. After binding the native, solubilized photosystem II (PSII) to Ni-NTA agarose the D1 assembled components were washed away by using 8 M urea and 0,3 % SDS. Isolated D1 already can be detected by Coomassie staining after SDS-PAGE. The eluted D1 protein contained as an impurity only the D2-protein, which was cross linked to D1. Using hydrogen peroxide it is shown, that the purified D1 protein can be employed to restriction analysis in vitro. Restriction fragments are easily detected by silver staining-instead of antibody reactions.

Key words: *Chlamydomonas reinhardtii, D1 protein; Photosystem II; His-tag.*

Щоб зробити білок D1 доступним для дослідження та аналізу змін, чистий препарат D1, який здійснює накопичення білка D1 швидким та відтворювальним методом, є необхідним. Дана робота представляє цей метод. Внутрішня структура білка D1 chlorophycee Chlamydomonas reinhardtii була включена в цикл D-e. Після зв'язування рідної, солюбілізованої фотосистеми II (ФС) для Ni-NTA агарози білка D1 зібрані компоненти були змиті за допомогою 8 М мочевини та 0,3 % SDS. Ізольовані білки D1 можна виявити за допомогою фарбування в Кумассі після SDS-PAGE. Вимивається білок D1, який міститься у вигляді домішки тільки білка D2, який був зв'язаний з D1. Використання перекису водню показали, що очищений білок D1 може бути використаний для обмеження аналізу в пробірці. Обмежені фрагменти легко виявити на срібному забарвленні – замість реакції антитіл.

Ключові слова: *Chlamydomonas reinhardtii, білок D1, фотосистема II, His-tag.*

Чтобы сделать белок D1 доступным для исследования и анализа изменений, чистый препарат D1, который осуществляет накопление белка D1 быстрым и воспроизводимым методом, является необходимым. Данная работа представляет этот метод. Внутреннее содержание белка D1 chlorophycee Chlamydomonas reinhardtii было включено в цикл D-e. После связывания родной, солюбилизированный фотосистемы II (ФС) для Ni-NTA агарозы белка D1 собраные компоненты были смыты с помощью 8 М мочевины и 0,3 % SDS. Изолированные белки D1 можно обнаружить с помощью окрашивания в Кумасси после SDS-PAGE. Вымывается белок D1, содержащийся в виде примеси только белка D2, который был связан с D1. Использование перекиси водорода показали, что очищенный белок D1 может быть использован для ограничения анализа в пробирке. Ограниченные фрагменты легко обнаружить на серебряной окраске – вместо реакции антител.

Ключевые слова: *Chlamydomonas reinhardtii, белок D1, фотосистема II, His-tag.*

Abbreviations

D1, D2	reaction center proteins of photosystem II
Del	*psb*A deletion mutant
IL	intronless *psb*A mutant
PSII	photosystem II
PSI	photosystem I
ROS	reactive oxygen species
DoDM	*n*-dodecyl β-D-maltoside

Introduction

Since many 100 of millions of years green plants use photochemical energy to transform water and CO_2

into glucose and oxygen inside chloroplasts. The primary charge separation takes place at the so called photosystem II (PSII). This multi protein complex, which is integrated into the thylakoid membrane, forms a dimer. Each monomer consists of 25 subunits and 57 different cofactors (Lucinski and Jackowski, 2006). The core complex, built from protein D1 and D1, is bound form the proteins CP43 and CP47. At the stromal side the light harvesting complex II (LHCII) is associated to PSII. It absorbs the irradiated light and forwards it to the primary electron acceptor P_{680}. This chlorophyll dimer is bound to the histidin residues His-198, respectively His-197 of the proteins D1 or D2. After activation it transmits electrons to the D1 bound pheophytin.

During charge separation reactive oxygen species (ROS) are produced. ROS are reacting with targets from their surroundings: for example leading to D1 cleavage. The damaged D1 protein is degraded by proteases, removed and replaced by a functional copy. Therefore it is known as that chloroplast protein showing the highest turnover.

To identify the occurring reactions inside of PSII and in that involved components different PSII-isolation methods were established.

Diner and Wollman (1980) described a preparation, which was used to isolate active PSII-particles from *Chlamydomonas reinhardtii* without contamination of PSI. Generating a sucrose gradient they isolated – without the additive of detergents – photosynthetically active membranes. These membranes were solubilized by digitonin and triton X-100 and separated again by a sucrose-gradient. An active PSII complex was isolated at a density of 24 % sucrose. Using a DEAE-Sephadex A-50-column and consequently removing five proteins the specific activity was improved about 50 %.

At the first time in 1981 a method which enabled the isolation of PSII from higher plants without PSI contamination was published by Berthold *et al.* (1981). Two solubilizing steps including a triton X-100 containing buffer were added to the membrane preparation of Robinson *et al.* (1980). The received PSII particles were stored using a sucrose-HEPES buffer.

Nanba and Satoh (1987) established a method for isolation of a complex consisting of D1, D2 and cytochrom b559. That one is based on the protocol developed by Kuwabara and Murata (1982). PSII particles were solubilized using a 4 % triton X-100 containing TRIS-buffer. Afterwards protein micelles were absorbed by a DEAE-Toyopearl 650S-column. At first associating proteins were washed away and then the D1/D2/Cytb559 complex was eluted using a salt gradient.

Tang *et al.* (1990) added further solubilization and chromatography steps to that from Nanbah and Satoh published protocol to isolate a D1/D2-complex out of *Spinacea oleracea*. The purified proteins occurred as a dimer. Neither D1, nor D2 monomer was detected. Tang *et al.* purified only cross linking products. Basing on these methods many other techniques were described for PSII separation (Shim *et al.* 1990, Shipton and Barber, 1991, Miyao, 1994, Schiller and Dau, 2000).

Sugiura, Inoue and Minagawa employing *Chlamydomonas reinhardtii* to create a D2 protein, which possesses a 6-fold, carboxy-terminal His-Tag (Sugiura *et al.* 1998). Utilizing a nickel resin column they isolated the His-labeled D2-protein together with the associated PSII proteins – such as D1, CP43, CP47 – by raising the imidazol concentration. They tried to label also the D1 protein of *Chlamydomonas reinhardtii* – using a His-tagged amino-terminus – but no photoautotrophically transformants were obtained. A native PSII was also isolated by using a 6-fold His-taged CP43 (Sugiura and Inoue, 1999), a 6-fold His-taged CP47 (Suzuki *et al.* 2003) and a 6-fold His-taged PsbH-protein (Cullen *et al.* 2007).

All these preparations are made to isolate the whole PSII complex or PSII-subspecies. To investigate modifications of only one PSII protein, the examined one had to be prepared with a high purity, well separated from other PSII components, and also in a quantity, to allow different studies. None of hitherto published protocol fulfills these criteria.

The current work presents a method to purify a tenfold His-tagged D1 protein from *Chlamydomonas reinhardtii* using Ni-NTA agarose. This method needs only 400 ml of algae for purification of 88 µg D1 protein in only 4 hours. Moreover, the high concentrated D1 protein shows a high purity. Therefore due to these characteristics it is suitable for modification and restriction analysis. Such a method is hardly needed, because the D1 protein, localized inside of the thylakoid membrane, is the most important target of oxidative attacks (Aro *et al.* 1993). During light exposure charge separation continuously takes place. That leads to a D1-damage coursed by reactive oxygen species. Here it is demonstrated that – using the described new D1 purification method – development of D1-fragments coursed by hydrogen peroxide can be easily detected by silver staining *in vitro*.

Material and methods

Cell culture

C. reinhardtii strain Del1 (Preiss *et al.* 2001) was grown on sterile TAP agar (Gorman and Levine, 1965) at a light intensity of 20 µmol of photons $m^{-2}s^{-1}$. After transformation, photosynthetic active strains were cultured on sterile HS agar (Sueoka, 1960) or grown in 400 ml glass flasks filled with TAP media at 25 °C and at a light intensity of 150 µmol of photons $m^{-2}s^{-1}$.

Construction of mutants

Escherichia coli strain DH5α was used as recipient strain for DNA transformation. Cells were grown in LB agar and in further case in LB media at 37 °C (Sambrook *et al.* 1989).

The plasmid pSH8 contained the intron-less *psbA*-Sequence of *Chlamydomonas reinhardtii*. The *Bst*EII site was used for pasting a tenfold His-tag via oligonucleotide linker (Fig. 1) into *psbA*. Synthetic oligomeres (Biomers) were phosporylated by T4 polynucleotidekinase at a concentration of 1 µg per 10 µl in buffer (Fermentas) containing 1 mM ATP for 1 hr at 37 °C. One-tenth of the same preparation

was ligated to plasmid (1-5 µg) in the same buffer overnight at 4 °C before purification by agarose gel electrophoresis. Restriction enzymes were used according to the manufacturs instructions (New England Biolabs). Typically 90 µg of DNA in 30 µl of the recommended buffer was digested with 1 unit of enzyme at the recommended temperatures. Cohesive ends of DNA were filled in with the GoTaq (Promega) in buffer (Promega) containing the four desoxyribonucleotides (1mM each). The transformation of strain DH5*α* was carried out according to Hanahan (1983). The plasmid preparation was performed by the method of Quiagen.

Biolistic transformation

C. reinhardtii mutant His10, that possesses a tenfold His-tag inside of PsbA, was created by using the particle gun (Klein *et al.* 1987) as described by Dauvillee *et al.* (2004). As carrier for precipitated PCR products Wolfram was employed. After particle bombardment cells were left on TAP agar and one day later placed on HS agar for selecting on photoautotrophic growth.

RNA analysis

Isolation of RNA was carried out as described by invitrogen (TRIzol®). Total RNA was separated on 1,2 % formaldehyde/ agarose gels as described by Sambrook *et al.* (1989) and Northern blot analysis was done according to Kroczek (1993). For detection of *psbA*-mRNA a Dig-labeled probe was used.

D1 purification

400 ml *C. reinhardtii* cells with a density of 4 × 10^6 cells/ml were harvested by centrifugation for 10 min at 4 000 *g*, resuspended in 0,1 M Na_2CO_3 and broken for two times by Yeda press treatment (Shneyour and Avron, 1970). Cell wall particles and unbroken cells were removed by centrifugation at 1 000 *g*. Thylakoid membrane was received after centrifugation at 20 000 *g* and resuspended in ice cold 0,1 M Na_2CO_3. Chlorophyll concentrations were determined by the method of Arnon (1949). Thylakoid membrane material of strain His10 comparing to 450 µg chlorophyll was used for saturating 2 ml Ni-NTA agarose (Quiagen). After solubilization of thylakoid membranes from His10 in 25 mM HEPES/NaOH (pH 7,5), 100 mM NaCl, 10 % glycerol (v/v), 2 % *n*-dodecyl *β*-D-maltoside (DoDM) (w/v) for 1 h at 4 °C and centrifugation at 13 000 *g* supernatant was loaded on Ni-NTA-Agarose as described by Sugiura *et al.* (1998). Washing the loaded material was performed by 25 mM HEPES/ NaOH (pH 7,5), 100 mM NaCl, 10 % glycerol (v/v), 0,03 % DoDM (w/v) and 15 mM imidazol. Thereafter at first 8 M urea and then 0,3 % SDS (w/v) was added to the buffer. The column was washed till the flow through had no visible impurities. The D1 protein was eluted with 40 mM MES/ NaOH (pH 6,0), 100 mM NaCl, 0,03 % DoDM (w/v), 10 % glycerol and 200 mM imidazol and concentrated by using a centriprep centrifugal column with Amicon Ultra-4 centrifugal filter devices with low-binding Ultracel membranes to get 1,8 µg D1 protein/ ml.

Protein analysis

Isolated proteins were solubilized for 5min at 95 °C with a sample buffer containing 5 % *β*-mercaptoethanol (v/v) (Laemmli, 1970) and separated by SDS-PAGE (Schägger *et al.* 1985) generally using 15 % polyacrylamide gels (14 × 14 × 0,75 cm). Gels were loaded on an equal protein basis. After electrophoresis, the proteins were electro blotted onto nitrocellulose according to Towbin et al. (1979) and incubated with antibodies as described (Preiss *et al.* 2001) or also stained. The staining procedure with Coomassie brilliant blue R250 was performed according to the instruction of Merril (1990). For silver staining the method of Berkelman and Stenst-edt (1998) was used.

To identify stained proteins, corresponding lanes were cut out of Coomassie blue stained gels and sent to Toplab.

H_2O_2 treatment

Isolated D1 protein was treated with 0,3-3 % H_20_2 (v/v). For inhibition of restriction 0,3 mM EDTA was added. The sample was incubated for 0-60 min or also 24 h at 4°C or 37°C inside of a thermo block.

Results

Transformation of *C. reinhardtii*

In order to isolate and purify the D1 subunit of PSII by affinity column chromatography methods, a mutant of *Chlamydomonas reinhardtii* was constructed by inserting an oligonucleotide linker encoding a tag with ten consecutive His residues into the intronless *psbA* gene (Fig.1) (Preiss *et al.* 2001). That mutant was named His10.

Since the outmost N- and C-termini of the mature D1 protein appear to be of functional importance (Nixon *et al.*, 1992; Takahashi *et al.*, 1996; Sugiura *et al.*, 1998), for the affinity tag a position within the extended D-d-e loop region of the stromal loop were chosen. Although the D1 protein sequence is highly conserved it has been shown that the loop region tolerates insertions without significantly affecting photoautotrophic growth (Kless and Vermaas, 1995). At the DNA level, suitable insertion positions were predetermined by unique *BstE*II restriction site within the intronless *psb*A gene (Fig. 1b). According to the 3-dimensional structure of the D1 protein, the affinity tag will thus be located in exposed regions facing the stroma side of the thylakoid membrane (Fig. 1c).

To generate transgenic chloroplasts expressing the affinity tagged D1 protein, in the current work chimeric genes containing the oligonucleotide insert were constructed (Fig. 1a, b).

Using the biolistic transformation method, the His10 construct was delivered into chloroplasts of recipient strain Del1 which lacks a 425 bp fragment within the *psb*A gene and is thus unable to grow photoautotrophically (Preiss *et al.*, 2001). Upon subsequent selection on minimal media allowing photosynthetic growth only, primary transformants were analysed by PCR for gene integration. Positive transformants were further propagated under selective conditions until homoplasmic lines were obtained.

Linker His10:
5′ – GT TAC CAT CAT CAT CAT CAT CAT CAT CAT CAT CAT G – 3′
3′ – GAT GTA GTA GTA GTA GTA GTA GTA GTA GTA CCA ATG – 5′

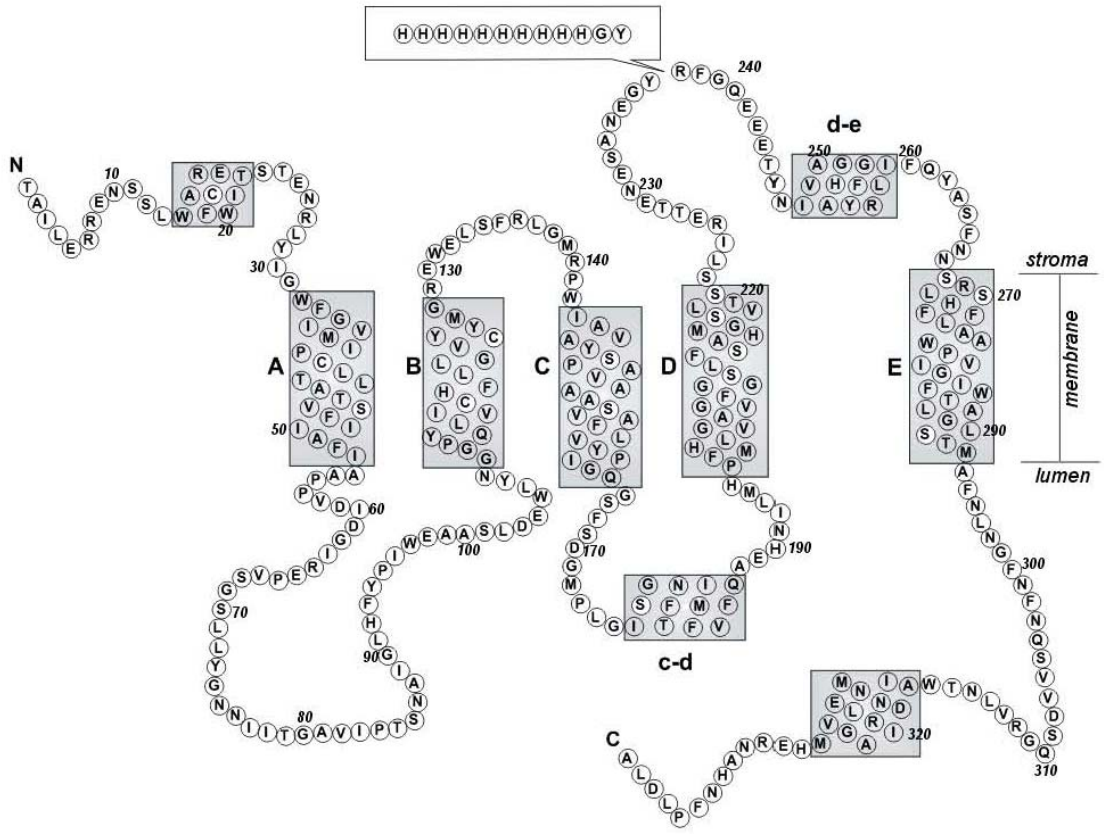
a)

Insertion:
5′ – (691) GAA TCA GCT AAC GAA GGT TAC | CGT TTC GGT CAA GAA – 3′
E S A N E G Y | R F G Q E

His10 CAT CAT CAT CAT CAT CAT CAT CAT CAT CAT GGT TAC
H H H H H H H H H H G Y

b)

Resulted protein:



c)

Fig. 1. Construction of the *Chlamydomonas reinhardtii* mutant His10. **(a)** For construction of His10 mutant the sequence of a tenfold His-tag was included into an oligonucleotide linker. **(b)** That one was inserted into the *psbA* sequence using the *Bst*EII restriction side. **(c)** After integration of the modified gene into the plastom of *Chlamydomonas reinhardtii* via particle bombardment a mutant was received, that expressed a D1 protein carrying a tenfold His-tag at amino acid position 237 inside of the d-e loop

Single homoplasmic strain from the transformation experiment was named His10 and further analyzed (Fig. 2). A PCR analysis with primers flanking the oligonucleotide insertion site identified a slightly larger band compared with the control strain IL and confirmed homoplasmicity (data not shown). DNA sequence analysis confirmed the correct in-frame insertion (data not shown). Northern blot analysis of total RNA revealed that the *psbA* mRNA is slightly larger in the transformants but accumulate to similar levels in IL and transgenic strain, indicating that the transgene does not alter *psbA* mRNA accumulation (Fig. 2a). Western blot analysis using D1 antiserum indicates similar protein accumulation in His10 strain (Fig. 2b).

Growth rate and oxygen evolution rate of His10 and the reference strain were measured, to investigate, that insertion of a tenfold His tag shows no negative effect to vitality of the His10 mutant.

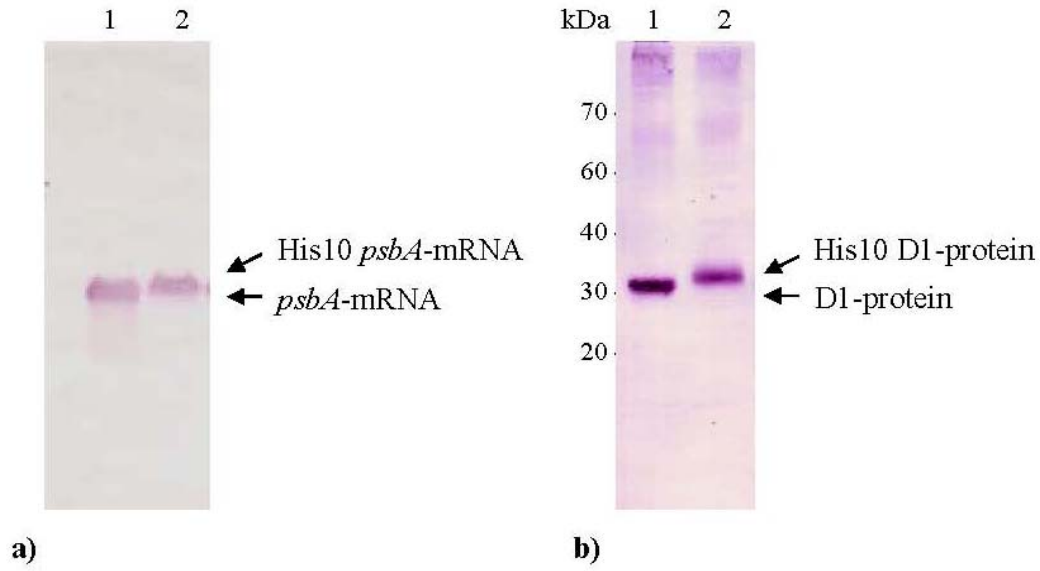


Fig. 2. Comparison of *psbA*-mRNA and PsbA-protein from reference strain Il and strain His10. **(a)** Isolation of total RNA and whole cell-protein was done after growing the used strains under standard conditions. *psbA*-mRNA of reference strain Il (lane 1a) and mutant His10 (lane 2a) was detected by Northern blot using a Dig labeled psbA-probe. **(b)** D1 protein of reference strain Il (lane 1b) and mutant His10 (lane 2b) was identified by immunoblotting of whole cell protein corresponding to 7 µg chlorophyll and incubation with antibody D1 global (Agrisera)

1,5 × 10^7 cells of strains Il and His10 were used, to inoculate kniese tubes and bubbled continuously with a mixture consisting of 5 % CO_2 and 95 % air at 70 µmol/m^2s and 72 °F. Numbers of cells were determined in regular intervals at OD_{750}. Growth curve was drawn up during 90 h (Fig. 3). Compared with mutant Il (set 100 %) for strain His10 a growth rate of 93 % was ascertained.

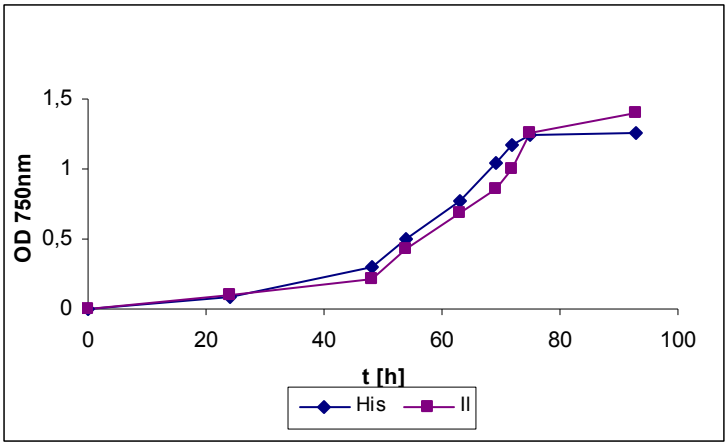


Fig. 3. Growth curves of reference strain Il and strain His10. Growth curves of Il and His10 respectively were measured at OD_{750} (OD: optical density) in fixed time (t) intervals [h]. Cells were grown in HS-medium at 70 µmol/m^2s and 72 °F and bubbled with a mixture consisting of 5 % CO_2 and 95 % air

For determination of photosynthesis rate cells of mutants Il and His10 grown in TAP medium, corresponding to 15µg chlorophyll were transferred to a Clark electrode. Oxygen evolution was measured per hour and mg chlorophyll. Using standard conditions a net-photosynthesis rate of 196,8 µmol O_2/h*mg Chl for mutant His10 and of 160,6 µmol O_2/h*mg Chl for reference strain Il were measured. The net-photosynthesis rate of strain His10 corresponds to 82 % to that of reference strain (set to 100 %).

Summarizing results obtained by measuring growth rate and oxygen evolution of mutant His10 and reference strain Il it can be concluded, that integration of a ten-fold His-tag inside of d-e loop of D1 protein has no significant effect on vitality.

Isolation of PSII complex

To isolate the whole, to the D1 protein associated PSII complex, the protocol of Sugiura *et al.* was used (1998). Thylakoid membranes were isolated by using Yeda press. Solubilization of membranes by a DoDM containing buffer followed. The solubilized protein was applied to Ni-NTA agarose column and washed with imidazol containing buffer till no protein was detected inside of flow through. Whole PSII complex was eluted by raising the imidazol concentration. The received fractions were analyzed by silver staining

(Fig. 4a) and by immunreaction (Fig. 4b). The flow through and the washing-steps consisted of thylakoid-membrane proteins such as LHCII, while PSII components were completely absent. The eluted PSII contained no LHC – but D1, D2, CP43 and CP47 were detected by silver staining and immunreaction, showing that it was possible to elute PSII by using a tenfold His-tagged D1 protein.

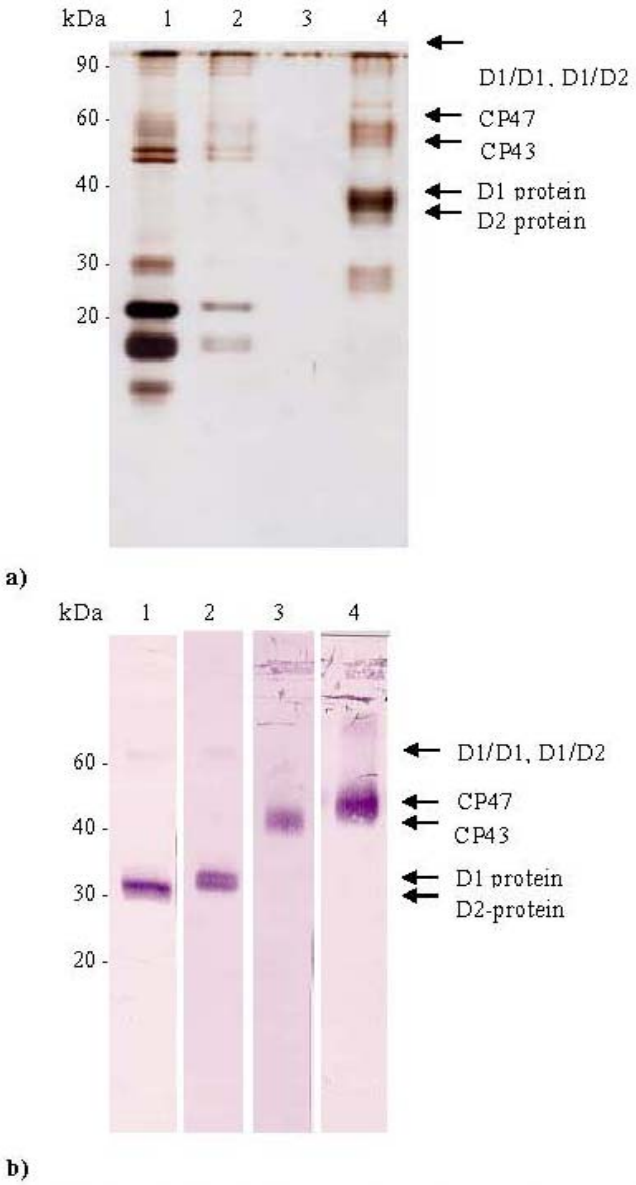


Fig. 4. Native elution of PSII complex using a His-tagged D1 protein. **(a)** After applying solubilized membranes to a Ni-NTA agarose column, the column was washed till no protein was detected inside of flow through (lane 1-3). Then the PSII complex (lane 4) was eluted by raising the imidazol concentration. 8 µl of the flow through and 2 µg protein of the eluted PSII complex were separated using SDS-PAGE (applied) and silver stained. **(b)** PSII core proteins D1 (lane 1), D2 (lane 2) as well as CP43 (lane 3) and CP47 (lane 4) inside of the eluted PSII fraction were determined by immunreaction

Isolation of D1 protein

For elution of the D1 protein at first the whole, by DoDM containing buffer solubilized PSII complex was bound to a Ni-NTA agarose-column as described by Sugiura *et al.* (1998). Afterwards column was washed by using a 15mM imidazol containing buffer, till the flow through had no visible impurities. Now D1 associating proteins were removed using one column volume 8 M urea wash buffer and then 0,3 % SDS (w/v) containing wash buffer until no protein was detectable inside of the flow through. Leaving out the urea washing step leads to an inefficient removal of associated PSII proteins. We also examined 2-6 M urea, 1-2 M NaCl, 20 % EtOH (v/v) and 40 % glycerol (v/v) for enabling PSII destabilization, but these substances had no effect on complex integrity.

After removal of peripheral associating proteins the D1 protein was eluted by raising imidazol concentrations. To get a high concentrated D1-solution the volume was reduced by using a 5 kDa size exclusion column. Received, on SDS-PAGE separated D1 protein, was stained using silver or also Coomassie blue. We

detected two protein lanes: one with a size of 30 kDa (Fig. 5) and one with 60 kDa. Both of them were analyzed by MALDI MS/MS. The 30 kDa large lane contained only peptides of the D1 protein (Fig. 6). But the 60kDa large lane consisted of the D1- and D2-protein. Immunreactions showed also in the 60 kDa area a contamination of the D2 protein, while the other PSII-components were completely absent (data not shown).

To verify that there are also D1 homodimers inside the 60 kDa area the D1 protein was eluted from out of the 30 kDa area and separated it again using the SDS-PAGE. As result we got again two lanes: one corresponding to 30 kDa and one shifting into the 60 kDa area. The hydrophobic helices of that membrane protein interacted in spite of employing SDS sample buffer.

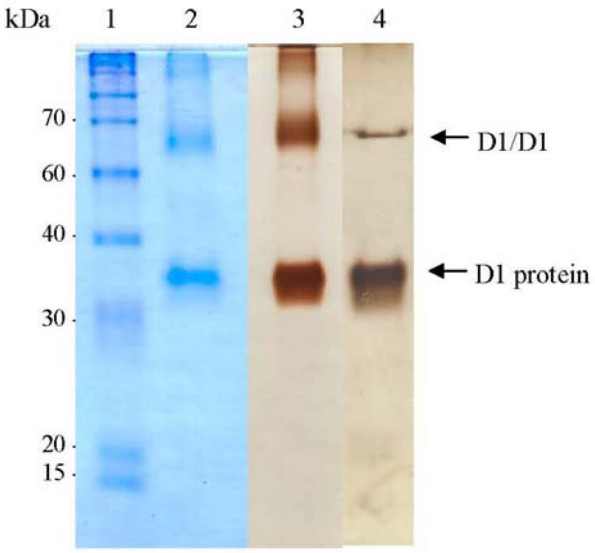


Fig. 5. Isolation of a tenfold His-tagged protein. PSII-complex was bound to Ni-NTA agarose according to Sugiura *et al.* (1998). Afterwards to D1 protein associated proteins were washed away by using a washing buffer containing at first 8M urea and then 0,3 % SDS. The pure D1 protein was eluted by raising the imidazol concentration and concentrated. 4µg D1 protein was applied to SDS-PAGE and after separation stained with Coomassie blue (lane 2) or also silver (lane 3). The Coomassie blue stained protein was eluted from PAGE, subsequently separated by SDS-PAGE again and silver stained (lane 4). For determining the protein size a protein standard (invitrogen) was loaded parallel (lane 1)

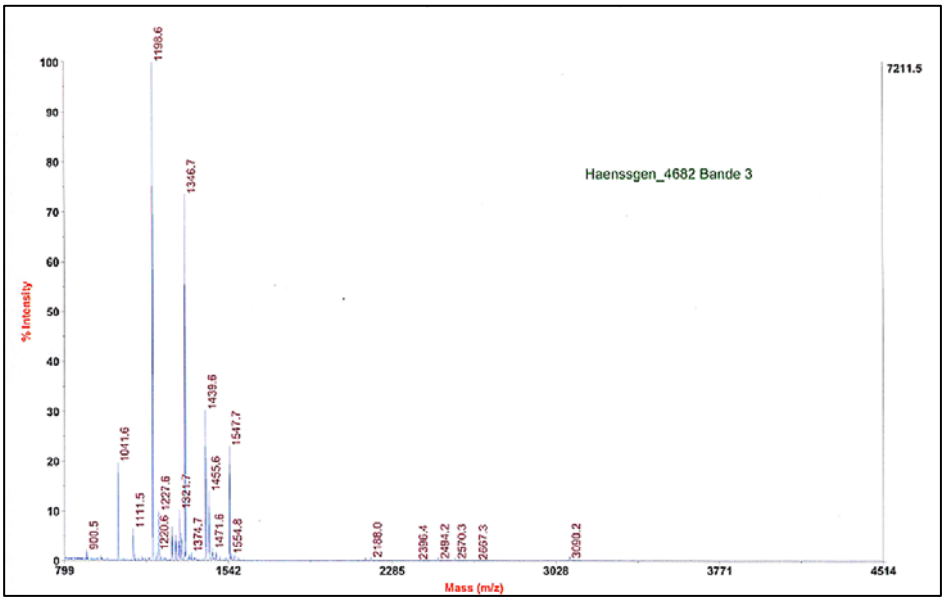


Fig. 6. MS/MS spectrum of trypsin cleaved completely oxidized D1 protein. Investigation of the 30 kDa large D1 protein lane by using MALDI-TOF identified the following peptides: ENSSLWAR, ANLGMEVMHER, VLNTWADIINR, FCEWITSTENR, LIFQYASFNNSR, FGQEEETYNIVAAHGYFGR. We found only peptide sequences of the hydrophilic loops and were not able to determine a sequence of the transmembrane helices. These peptides were linked by hydrophobic interactions and therefore could not be applied to TOF

Using 400 ml algae suspension, being in half logarithmic growth rate, thylakoid membrane material corresponding to 13 040 µg chlorophyll$_{a+b}$ (230 mg whole cell protein) was obtained. Starting with that quantity of whole cell protein we got 440 µl suspension containing 0,2 µg D1 protein/µl.

H_2O_2-cleavage of D1 protein

Isolated D1 protein was treated with hydrogen peroxide *in vitro* to simulate the effect of that ROS to the *in vivo* during light exposure by ROS damaged membrane protein.

At first we incubated 2 µg pure D1 protein with 0,03-015 % H_2O_2 (v/v) for 2 h at 37 °C – in the presence of 2 mM EDTA to prevent complete restriction (Fig. 7). After that treatment the protein was separated by SDS-PAGE and silver stained.

A 24 hour hydrogen peroxide incubation of D1 protein led to building of D1 cleavage products (Fig. 8). Increasing temperature from 4 °C to 37 °C a trifle increase of the reaction velocity was obtained. After addition of 2mM EDTA more cleavage products possessing a defined molecular weight occurred inside of 20 kDa range. During 24 hours the chelator prevented the complete degradation of D1, which was also received in the presence of just 0,03 % H_2O_2 (v/v). In the absence of EDTA, the D1 protein underwent a stronger shift to the higher molecular weights. Also building of many fragments having a very different size was noticed.

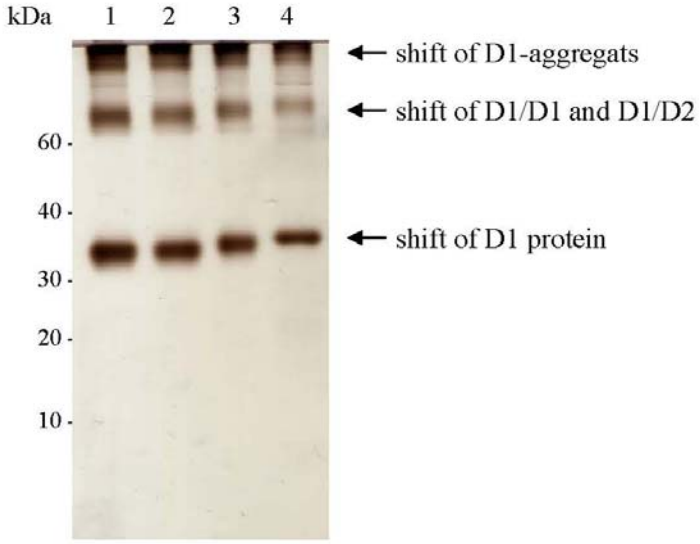


Fig. 7. H_2O_2-incubation of isolated D1 protein. 2µg D1 protein was treated for 2 h at 37°C with 0,03 % H_2O_2 (v/v) and 2 mM EDTA (lane 2); with 0,09 % H_2O_2 (v/v) and 2 mM EDTA (lane 3) as well as with 0,15 % H_2O_2 (v/v) and 2 mM EDTA (lane 4). D1 protein that was treated with 2 mM EDTA (2h at 37°C) was used as reference (lane 2). After separation on SDS-PAGE, the proteins were stained using silver staining

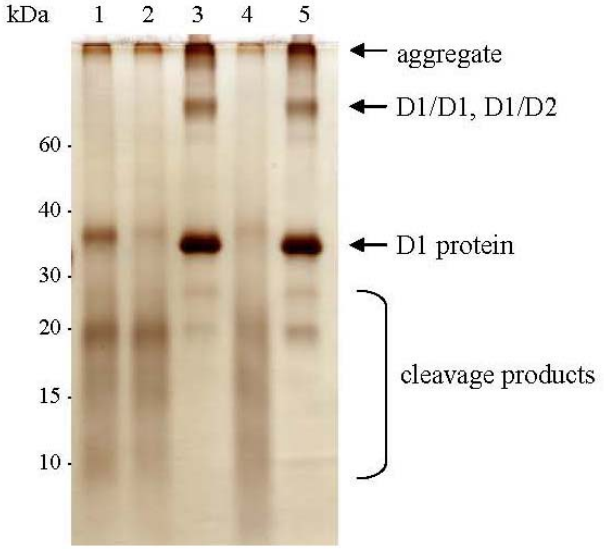


Fig. 8. Cleavage of D1 protein coursed by H_2O_2. Cleavage of 2 µg D1 protein was induced by hydrogen peroxide incubation within 24 h at different conditions. D1 incubation was done using 0,3 % H_2O_2 (v/v) at 4 °C (lane 1), 3 % H_2O_2 (v/v) at 4 °C (lane 2), 3 % H_2O_2 (v/v) and 2 mM EDTA at 4 °C (lane 3), 0,3 % H_2O_2 (v/v) at 37 °C (lane 4), 3 % H_2O_2 (v/v) and 2 mM EDTA at 37 °C (lane 5). After separation on SDS-PAGE, the proteins were detected using silver staining

Conclusion

Construction of His-tagged D1 mutant

The D1 protein is an integral thylakoid membrane protein, which consists of five transmembrane α-helices. Different cofactors which take part on the photosynthetically process are bound to D1 protein. The N-terminus is stromal exposed, while the C-terminal extension towers into the lumen. They are needed for integration into the thylakoid membrane, maturation and for degradation by proteases in a not yet completely understood manner. Together with D2-protein it builds the core of PSII. Taking part in electron transport and therefore being an important target of oxidative damage it is that protein in chloroplasts which shows the highest turnover under illumination. For the investigation of D1 maturation and D1 cleavage after damage a preparation is needed which allows purification of that membrane protein in quantity and without impurities.

We inserted a tenfold His-tag into the stromal exposed d-e loop between the amino acids Trp 237 and Arg 238. The amino acids Gly and Trp were doubled because of the insertion. In contradiction to the theory of Sugiura *et al.* (1998) the obtained homoplasmic mutant His10 expressed an intact, functional D1 protein. *psb*A mRNA and PsbA content from His10 mutant were nearly identically to that of reference strain IL. Also growth rate and net photosynthesis were only poor deteriorated, in comparison to the reference strain. These results underlay that we got a completely functional His-insertion mutant.

Isolation of His-tagged D1 protein and of PSII complex

Following the example of Sugiura *et al.* (1998), Sugiura and Inoue (1999), Suzuki *et al.* (2003) and Cullen *et al.* (2007) we labeled the D1 protein with a His-tag in order to isolate the labeled protein by IMAC. Expressing a tenfold – instead of a six fold – His-tag raised the binding efficiency to metal affinity matrix.

Solubilized thylakoid membrane material from mutant His10 was loaded onto Ni-NTA agarose according to Sugiura *et al*. (1998). His-labeled D1 protein was building a complex together with the nickel ions of the matrix. After removal of unbound material the whole PSII complex was eluted. Unspecific bound proteins were excluded because of Ni-NTA saturation with PSII material. These results were used to develop a D1 isolation method. At first we denatured the photosynthetic membranes and bound the received protein extract to Ni-NTA agarose. That resulted in a D1 protein preparation showing many impurities, while the made PSII preparation according to Sugiura *et al.* (1998) showed no presence of proteins, which are not included in the PSII apparatus and was also absent of LHCII proteins. That means that saturation of Ni-NTA agarose by the PSII complex avoided contamination of the foreign protein. Therefore, in following studies we bound the whole PSII complex to Ni-NTA agarose using the tenfold His-tagged protein and thereafter washed the complex with different substances to remove the D1 associated proteins completely.

At first NaCl was added to washing buffer to prevent ionic interactions. Addition of ethanol and of an increased concentration of glycerol inside of washing buffer interfere hydrophobic interactions between membrane proteins. But these chemicals showed no effect to the composition of PSII complex. Removal of peripheral to D1 associated components was attained by adding of 8 M urea and thereafter addition of 0,3 % SDS (v/v) to the washing buffer. Using urea membrane proteins were unfolded and following exposed there hydrophobic areas up to solution (Herbert, 1999). As result SDS integrated non tagged membrane – or membrane associating – proteins inside micelles and departed them. If that washing step was done only with SDS and without urea inside the washing buffer a large part of proteins was removed. But after silver staining according to Berkelman and Stenstedt (1998) PSII components, being impurities, were detected.

Concentration of eluted D1 protein done by 3 kDa exclusion columns led to Coomassie blue-stainable crowds of that membrane protein. Our D1 preparation showed after separation on SDS-PAGE two protein lanes in gel. They were analyzed by immunreaction using D1, D2, CP43 and CP47 antibody and also by MALDI MS/MS. Only the 38,8 kDa large D1 protein was provable by immunreaction and MALDI TOF inside the 30 kDa area. Inside the 60 kDa area and also between separation and stacking gel two proteins – the D1 protein as well as the D2 protein – were identified. They occurred as heterodimers – due to growth conditions: the His10 mutant was cultured under normal light conditions (60 μmol/m^2s). According to Ishikawa *et al.* (1999) even weak illumination led to cross linking products of D1 and D2. Cross linking is covalent and irreversible.

Cutting the D1 protein out of the 30 kDa area and loading it again on SDS-PAGE we showed, that also homodimers of D1 are present.

D1 cleavage induced by hydrogen peroxide in vitro

Hydrogen peroxide courses stress which leads to damage of PSII (Allakhverdiev and Murata, 2004). After incubation of an electro eluted D1 protein together with the non-radical reactive derivate hydrogen peroxide Lupinkova and Komenda (2004) detected 24, 20, 5 and 9 kDa large D1 fragments using immunreaction. But in some cases samples, containing cleaved D1-products, are only hardly distinguished from samples which represent non cleaved D1 protein.

We illustrate that, using the presented D1 extraction method, pure D1 protein can be employed to investigate the differences between hydrogen peroxide cleaved and non cleaved D1 protein exactly. Incubation of D1 protein together with hydrogen peroxide and EDTA led to shifting of protein lane to higher molecular weights inside of SDS-PAGE during two hours. After 24h in the presence of EDTA distinct degradation fragments are observed, whereas the absence of EDTA led to nearly complete D1 degradation. That observation can be explained by the presence of metal ions inside of the sample. During so called «Fenton-reaction» iron and hydrogen peroxide reacting to iron(III), an hydroxyl radical and an hydroxyl anion. Hydroxyl radicals are able to induce radical reactions. In absence of chelators these radical reactions showing a high velocity and peptide bounds between amino acids of D1 are cleaved.

Adding low amounts of EDTA or also EGTA metal ions are complexed. Only a few ions are in solution and consequently able to initiate Fenton reactions. The results point out that the initial cleavage hydrophobic areas are discriminated and hydrophilic, solution exposed areas preferred. Instead of iron also low valent metal complexes as Cu(II), Ti(III), Cr(II), Co(II) or also Ni(II) are in position to react as Fenton reagent (Torreilles *et al.* 1990). If iron ions are removed from D1 during preparation nickel irons took off from nickel column undertake the function as Fenton reagent.

After thylakoid membrane isolation the D1 protein, associated proteins as well as components continue their further: LHCII leads electrons to PSII and OEE-proteins cleave water. Q_A is reduced, protoned and released. Thereby charge separation of primary radical pair P_{680}^+ – $Pheo^-$ is favored. That leads to building of triplet chlorophyll, which reacts with oxygen and produces singlet oxygen (Lupinkova and Komenda, 2004). That points out that the *in vitro* observed cleavage fragments can also occur *in vivo*.

In following studies we will investigate the comparison between *in vitro* occurring and *in vivo* observed degradation fragments using our established D1 preparation protocol.

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