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BRIEF COMMUNICATION

Pigment composition and functional state of the thylakoid membranes during preparation of samples for pigment-protein complexes separation by nondenaturing gel electrophoresis

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Abstract

The present study was conducted to examine changes in photosynthetic pigment composition and functional state of the thylakoid membranes during the individual steps of preparation of samples that are intended for a separation of pigment-protein complexes by nondenaturing polyacrylamide gel electrophoresis. The thylakoid membranes were isolated from barley leaves (*Hordeum vulgare* L.) grown under low irradiance (50 μmol m^{-2} s^{-1}). Functional state of the thylakoid membrane preparations was evaluated by determination of the maximal photochemical efficiency of photosystem (PS) II (F_V/F_M) and by analysis of excitation and emission spectra of chlorophyll *a* (Chl *a*) fluorescence at 77 K. All measurements were done at three phases of preparation of the samples: (*1*) in the suspensions of osmotically-shocked broken chloroplasts, (*2*) thylakoid membranes in extraction buffer containing Tris, glycine, and glycerol and (*3*) thylakoid membranes solubilized with a detergent decyl-β-D-maltosid. F_V/F_M was reduced from 0.815 in the first step to 0.723 in the second step and to values close to zero in solubilized membranes. Pigment composition was not pronouncedly changed during preparation of the thylakoid membrane samples. Isolation of thylakoid membranes affected the efficiency of excitation energy transfer within PSII complexes only slightly. Emission and excitation fluorescence spectra of the solubilized membranes resemble spectra of trimers of PSII light-harvesting complexes (LHCII). Despite a disrupted excitation energy transfer from LHCII to PSII antenna core in solubilized membranes, energy transfer from Chl *b* and carotenoids to emission forms of Chl *a* within LHCII trimers remained effective.

Additional key words: 77-K chlorophyll fluorescence spectra; *Hordeum vulgare*; photochemical efficiency; polyacrylamide gel electrophoresis; thylakoid membranes isolation.

Nondenaturing polyacrylamide gel electrophoresis is an important biochemical method of the photosynthesis research providing a deeper insight into the structure-function characteristics of the photosynthetic apparatus at the level of pigment-binding protein complexes (PPCs) of the thylakoid membranes. Separated PPCs can be used for the subsequent analyses in order to study *e.g.* pigment composition of the individual complexes (Thayer and Björkman 1992, Lee and Thornber 1995), assembly of the light-harvesting complexes of PSII during greening of the intermittent light-grown plants (Dreyfuss and Thornber 1994) or regulation of PSII antenna size and PSI/PSII ratio in plants acclimated to different environmental conditions (Ferraro *et al.* 2003, Ballottari *et al.* 2007).

The interpretation of findings obtained from native

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Abbreviations: Cars – carotenoids; Chl – chlorophyll; Dm – decyl-β-D-maltosid; F_V/F_M – maximal photochemical efficiency of photosystem II; LHCII – light-harvesting complexes of photosystem II; PPCs – pigment-protein complexes; PSI and PSII – photosystem I and II; RES1 and RES2 – resuspension medium 1 and 2; VAZ – the pool of xanthophyll cycle pigments (violaxanthin + antheraxanthin + zeaxanthin).
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gel electrophoresis is based on the assumption that they reflect the state of PPCs in intact leaves. However, little is known how preparation of the thylakoid membrane samples that are loaded onto a polyacrylamide gel affects pigment composition and the functional state of the thylakoid membranes and the individual PPCs. Generally, preparation of the samples to perform electrophoretic separation of PPCs consists of three basic steps. Firstly, after homogenization of the leaf tissue, filtration and subsequent centrifugation, the sedimented chloroplasts are osmotically shocked to rupture chloroplast envelope membranes. Secondly, the thylakoid membranes obtained by further centrifugation are suspended in an extraction buffer containing Tris, glycine, and glycerol (Peter and Thornber 1991). Tris and glycine are compounds of the electrode buffer used during electrophoresis and glycerol is used to keep samples at the bottom of the well in gel so that samples do not undergo convective mixing with the electrode buffer (Shi and Jackowski 1998). In the last third step the thylakoid membranes are solubilized using a suitable detergent in order to release PPCs from the membranes without a disruption of pigment-protein interactions.

Besides changes caused by the electrophoretic fractionation of PPCs itself, like release of pigments from their binding sites (Lee and Thornber 1995), changes occurred during preparation of the thylakoid membranes should be taken into account as well. The objective of the present study was to examine changes in pigment composition and the structure-functional state of the thylakoid membranes during the individual steps of sample preparation for separation of PPCs by nondenaturing polyacrylamide gel electrophoresis. The structure-functional state of the thylakoid membrane preparations was evaluated by determination of the maximal photochemical efficiency of PSII (F_V/F_M) and by the analysis of excitation and emission spectra of chlorophyll *a* (Chl *a*) fluorescence at 77 K.

Spring barley (*Hordeum vulgare* L. cv. Bonus) was grown from seeds under controlled environment conditions inside a growth chamber (*BioLine VB1014*, *Vötsch*, Germany) at the photosynthetic photon flux density of 50 μmol m^{-2} s^{-1}, 20°C, 65% relative air humidity and 16/8 h day/night regime. The middle segments of 8-day-old primary leaves were used for both measurements on the intact leaves and isolation of the thylakoid membranes.

Barley leaves were harvested for isolation of the thylakoid membranes after 2 h of dark period. The modified method described by Ilík *et al.* (2002) was used. The leaf segments (12 g) were homogenized for 10 s in a grinding medium using *Ultra-Turrax T25* homogenizer (*IKA Labortechnik*, Staufen, Germany). The grinding medium contained 0.33 M sorbitol, 5 mM $MgCl_2$, 10 mM NaCl, 2 mM sodium ascorbate, and 50 mM Tricine-NaOH, pH 7.8. The homogenate was rapidly filtered through four layers of polyamide sieve *Uhelon 130T* (*Silk & Progress*, Brněnec, Czech Republic) with mesh width of 42 μm and the filtrate was centrifuged at 3,020 × *g* for 2.5 min (*Sigma 3K 30*, Osterode, Germany). The sedimented chloroplasts were osmotically lysed by suspending the pellet in a resuspension medium consisting of 5 mM $MgCl_2$, 10 mM NaCl, and 50 mM Tricine-NaOH, pH 7.8 (denoted as RES1 medium). At this point of isolation procedure, the part of suspension was taken for the measurements. Such samples were denoted as RES1 samples. The thylakoid membranes were obtained by a subsequent centrifugation at 20,000 × *g* for 6 min. The pellet was resuspended in a resuspension medium that contained 11.3 mM Tris, 87 mM glycine and 9% (v/v) glycerol (denoted as RES2 medium). Again, the part of thylakoid membranes resuspended in RES2 medium used for the measurements were correspondingly denoted as RES2 samples. The remaining thylakoid membranes were solubilized with 20% (w/v) stock of decyl-β-D-maltosid (Dm). This surfactant and the RES2 membranes were mixed to obtain a final 2% concentration of the surfactant and Chl (*a*+*b*) concentration of 1,250 μg ml^{-1}. The extract was centrifuged at 7,500 × *g* for 2.5 min to remove a colorless insoluble material and to obtain a green supernatant (denoted as Dm medium) that was also used for the measurements. All isolation steps were performed at 0–4°C under dim green light. Immediately after preparation of each of the suspensions of thylakoid membranes that were kept in darkness on ice, the samples for pigment analysis and the measurements of F_V/F_M and Chl *a* fluorescence spectra at 77 K were taken. Chl (*a*+*b*) content of all suspensions was adjusted to 1,000 μg ml^{-1}. The results of six independent isolation procedures are presented. As a control, the intact leaves after 2 h of dark adaptation were used for the measurements.

The ratios of Chl *a* to Chl *b* (Chl *a*/*b*) and of total chlorophylls to total carotenoids [Chl (*a*+*b*)/Car (x+c)] were estimated spectrophotometrically (*UV/VIS 550, Unicam*, England) from the pigment extracts according to Lichtenthaler (1987). In the case of suspensions, the pigments were extracted in 80% acetone. Because the single extraction of pigments (mainly β-carotene) from leaves in aqueous acetone solvent is incomplete (Thayer and Björkman 1992, Dunn *et al.* 2004), we used triple acetone extraction (80:100:100%) of pigments from leaves. The contents of the individual carotenoids (lutein, neoxanthin, β-carotene, and the pool of xanthophyll cycle pigments, *i.e.* violaxanthin + antheraxanthin + zeaxanthin) expressed on a Chl (*a*+*b*) basis were estimated by the gradient reversed-phase high-performance liquid chromatography (*TSP Analytical*, USA) according to Färber and Jahns (1998) with a minor modification (Kurasová *et al.* 2003). The details are described in our previous study (Štroch *et al.* 2008).

Chl *a* fluorescence spectra at 77 K were measured using a luminiscence spectrophotometer *LS50B* (*Perkin–Elmer*, UK) equipped with the custom-made Dewar-type

optical cryostat. The emission spectra were recorded at the excitation wavelength of 436 nm (preferential excitation of Chl *a*) and the excitation spectra were measured at the emission wavelength of 685 nm (preferentially PSII emission). The emission spectra of the thylakoid membrane suspensions were measured with 5- and 2.5-nm slit widths of excitation and emission monochromators, respectively. The measurements of excitation spectra of the suspensions were carried out with 2.5- and 5-nm slit widths of excitation and emission monochromators, respectively. All measurements with the leaves were done with 5-nm slit widths of both monochromators. The emission spectra were corrected for the spectral sensitivity of the detection system. The excitation spectra were automatically corrected for the output of excitation source, the efficiency of excitation monochromator and the sensitivity of reference photomultiplier.

F_V/F_M $[(F_M–F_0)/F_M]$ was determined using a pulse amplitude-modulated fluorometer (*PAM 101/103, Walz*, Effeltrich, Germany) equipped with the emitter-detector unit *ED-101BL* employing a blue light-emitting diode as a source of excitation light with the maximum at the wavelength of 461 nm (Štroch *et al.* 2005). The fluorescence emission was detected above 660 nm. F_0 was obtained upon excitation with a weak measuring light modulated at 1.6 kHz that did not induce any significant variable fluorescence. For F_M determinations, the saturating "white-light" pulses of 0.8-s and 1-s duration were triggered using a *KL 1500* halogen lamp (*Schott*, Mainz, Germany) for the intact leaves and thylakoid membrane suspensions, respectively. The different length of saturating pulses was needed for correct estimation of F_M. The incident photosynthetic photon flux density of the applied pulses was approximately 5,000 μmol m^{-2} s^{-1}.

The statistical differences between the means were determined using a two-sample *F*-test for variances followed by a Student's *t*-test at three levels of significance (0.001, 0.01, and 0.05). Based on the results of the *F*-test, the *t*-test assuming either equal or unequal variances was used. All statistical tests were performed using the data analysis tools of *Microsoft Office Excel 2007*.

F_V/F_M of the thylakoid membranes suspended in RES1 medium after the first centrifugation was 0.815 ± 0.004, documenting a successful isolation procedure in the first phase of preparation of the thylakoid membranes. Nevertheless, F_V/F_M was slightly but significantly reduced from 0.826 ± 0.002 in the intact leaves (P<0.001). Despite this decrease of F_V/F_M, PSII in the isolated membranes can be considered to be still in a fully functional state. After the second centrifugation and subsequent resuspension of the sedimented thylakoid membranes in RES2 medium, F_V/F_M decreased by 11% to the value of 0.723 ± 0.018. This moderate impairment of PSII functional state was expected, as the physico-chemical properties of RES2 medium did not correspond to conditions needed to maintain an optimum functional state of the thylakoid membranes (Leegood and Malkin 1986). RES2 medium contained Tris, glycine, and glycerol, the compounds necessary for a subsequent PPCs separation by a native gel electrophoresis (Peter and Thornber 1991, Ilík *et al.* 2002). After solubilization of the membranes with decyl-maltosid, PSI and PSII supercomplexes underwent disassembly that resulted in a very low F_V/F_M in the range of 0.0 to 0.1 (data not shown).

Chl *a*/*b* showed a decreasing trend during the individual steps of thylakoid membranes isolation (Fig. 1*A*). The suspension of membranes in Dm medium exhibited a slight but statistically significant decrease of Chl *a*/*b* by 3.5% in comparison with the pigment extracts from intact leaves (P<0.01). This indicates that Chl *a* content diminished more than that of Chl *b*. Chl (*a*+*b*)/Car (x+c) was quite stable during individual steps of the thylakoid membrane preparation (Fig. 1*B*). The ratios of the individual carotenoids to Chl (*a*+*b*) also showed no pronounced changes during preparation of the thylakoid membranes (Fig. 1*C–F*). Thus, our data showed that pigment composition was not pronouncedly changed during preparation of the thylakoid membrane samples that are loaded onto a polyacrylamide gel in order to perform the electrophoretic separation of PPCs in a native state. While Cars content was not significantly altered, one could expect a slight loss of Chl *a*, especially during solubilization of the thylakoid membranes. It can be due to a partial loss of stromal thylakoids that break more easily during centrifugation and cannot be pelleted. Therefore, a loss of stromal thylakoids with much higher Chl *a/b* in comparison with grana thylakoids (Quiles *et al*. 1999) can result in decrease of Chl *a/b* in RES2 suspensions (Fig. 1*A*). In addition, decreased Chl *a/b* in Dm samples can be explained by the fact that efficiency of solubilization never reaches 100% and PSI with higher Chl *a/b* is the least solubilizable complex.

The maximum of 77-K Chl fluorescence emission originating from light-harvesting complexes of PSI (LHCI) in the thylakoid membrane suspensions was shifted from 743 nm observed in leaves towards shorter wavelengths by 3–4 nm (Fig. 2*A*). PSII emission bands with the maxima around 684 and 692 nm were significantly higher for the thylakoid membrane samples than for intact leaves. Higher PSII emission (relative to PSI emission band) together with the shift of far-red Chl fluorescence maximum to shorter wavelengths is a characteristic effect of decreased reabsorption of the emitted Chl fluorescence in the suspensions (Buschmann 2007). Thylakoid membranes suspended in RES1 medium showed slightly higher PSII emission as compared to RES2 membranes (significantly only at the maximum of 692 nm, P<0.01). Solubilization of the membranes resulted in a pronounced PSII emission band with the maximum at 679 nm (Fig. 2*A*). Andreeva *et al.* (2009) observed the maximum at 679 nm in isolated LHCII trimers. Other authors reported the maximum near

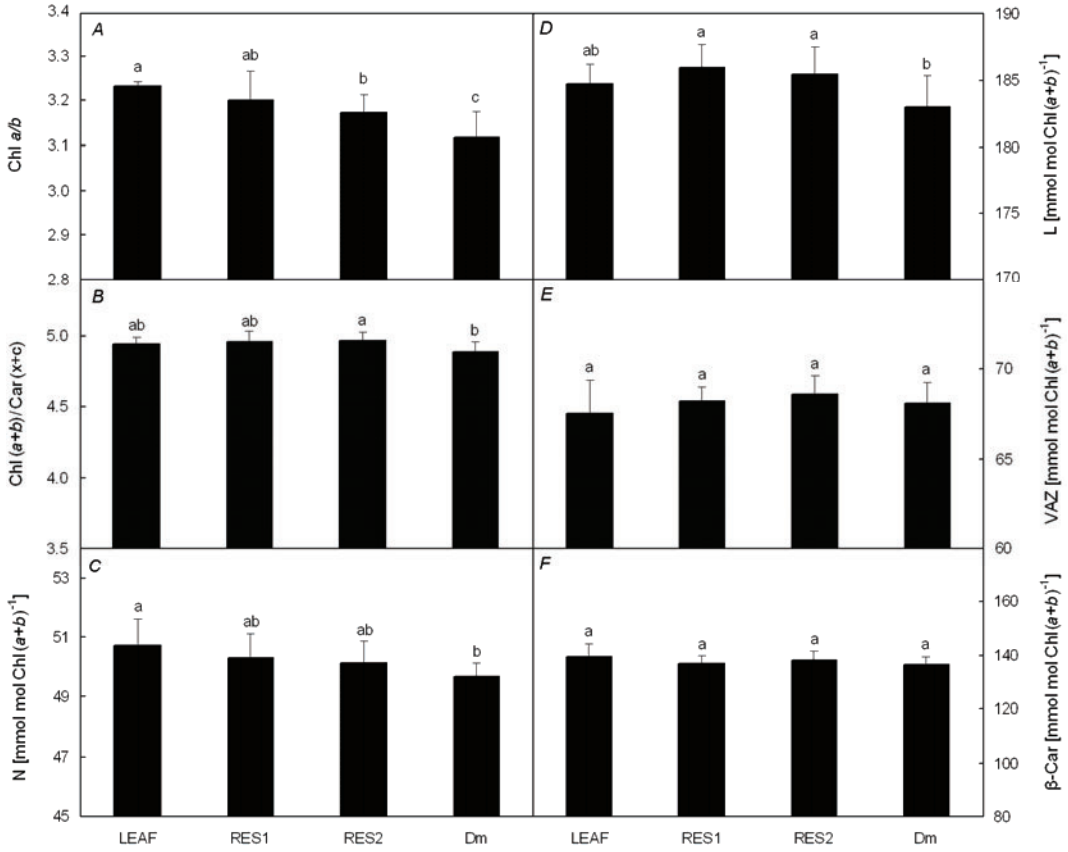

Fig. 1. *A*: The ratio of chlorophyll *a* to chlorophyll *b* (Chl *a/b*), *B*: the ratio of total chlorophylls to total carotenoids [Chl (*a*+*b*)/ Car (x+c)], *C*: the content of neoxanthin (N), *D*: lutein (L), *E*: xanthophyll cycle pigments (VAZ) and *F*: β-carotene (β-Car) expressed on Chl (*a*+*b*) basis. The pigment contents were determined in the extracts from *H. vulgare* leaves and isolated thylakoid membranes suspended in RES1, RES2 and Dm media during the individual steps of isolation procedure. Data followed by the same letter indicate non-significant difference (P>0.05; Student's t-test). $n = 6 \pm$ SD.

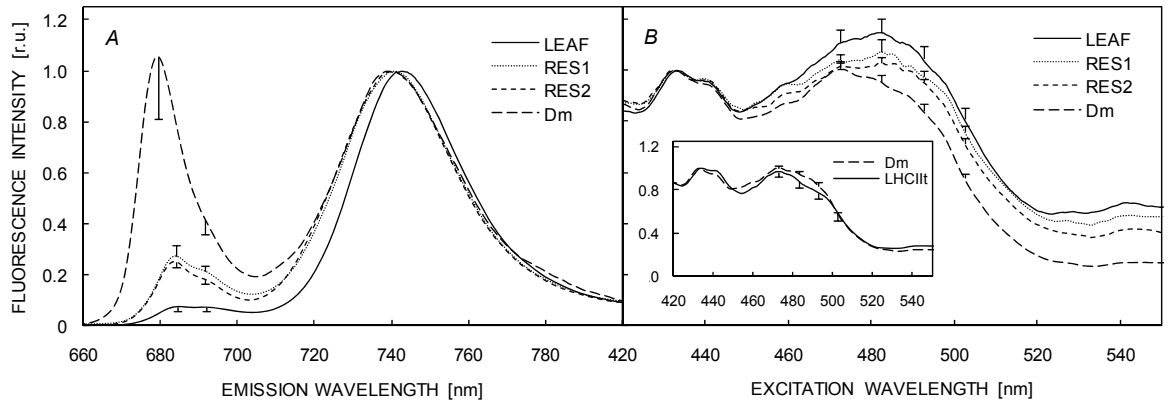

Fig. 2. *A*: Chlorophyll *a* fluorescence emission and *B*: excitation spectra at 77 K of *H. vulgare* leaves and isolated thylakoid membranes suspended in RES1, RES2 and Dm media during the individual steps of the isolation procedure. The *inset* shows excitation spectra of Dm membranes and band containing LHCII trimers (LHCIIt) obtained by nondenaturing gel electrophoresis according to Ilík *et al.* (2002). The emission spectra were recorded at the excitation wavelength of 436 nm and normalized at the long-wavelength maximum. The excitation spectra were measured at the emission wavelengths of 685 nm for leaves and the membranes and 681 nm for LHCIIt bands. The excitation spectra were normalized at the excitation maximum of chlorophyll *a* in the Soret region. The mean spectra from 6–12 samples are presented. *Error bars* indicate SD.

680 nm in LHCII trimers isolated after solubilization with Dm (Hemelrijk *et al.* 1992, Ruban *et al.* 1997). Thus, PSII emission band in Dm samples corresponds to emission from LHCII trimers that were detached from PSII core after Dm treatment. Pronounced emission of LHCII trimers indicates a disrupted excitation energy transfer to PSII antenna core, that can contribute to a strong F_V/F_M reduction.

In 77-K Chl fluorescence excitation spectra detected at PSII emission, the characteristic main excitation maximum of Chl *a* was observed at 433 nm for all samples (Fig. 2*B*). The broad Chl *b* and Cars excitation band in the spectral region of 460–510 nm with the maximum around 482 nm was detected for intact leaves, RES1 and RES2 samples. The maximum of this heterogeneous excitation band was shifted to 472 nm in Dm suspensions due to reduced contribution of excitation bands at longer wavelengths. The shape of excitation spectrum of solubilized thylakoid membranes was very similar to that of a band containing LHCII trimers obtained by nondenaturing gel electrophoresis (see *inset* of Fig. 2*B*).

The relative efficiency of excitation energy transfer from Chl *b* and Cars to the Chl *a* forms emitting PSII fluorescence can be estimated from the magnitude of Chl *b* and Cars excitation band relative to the excitation maximum of Chl *a* (Čajánek *et al.* 2002). In comparison with intact leaves, the efficiency of excitation energy transfer within PSII was slightly reduced in RES1, RES2 and more pronouncedly in Dm samples (Fig. 2*B*). Slightly decreased Chl *b* and Cars excitation band in RES2 suspensions relative to RES1 samples was accompanied by reduced F_V/F_M. However, it should be noted that the high efficiency of excitation energy transfer from Cars to Chl *a* emission forms at PSII is not a prerequisite for optimum F_V/F_M. The *chlorina f2* mutant of barley with impaired assembly of LHCII possesses almost the same PSII photochemical efficiency as the wild type barley, in spite of extremely low efficiency of excitation energy transfer from Cars to the PSII Chl *a* emission forms (Štroch *et al.* 2004). On the contrary to RES1 and RES2 samples, Cars excitation bands in the long-wavelength region (above 480 nm) were mainly reduced in Dm samples. As mentioned, in solubilized thylakoid membranes the PSII Chl *a* fluorescence was emitted from the LHCII trimers due to the disconnection of the excitation energy transfer to the PSII core. Thus, the reduction of the Cars bands can reflect either the lower content of the xanthophyll cycle pigments as compared to the inner LHCII complexes (Bassi *et al.* 1993) or the slightly decreased efficiency of excitation energy transfer from the xanthophylls to the fluorescing forms of Chl *a* within solubilized LHCII complex. In addition, a lower content of lutein (Fig. 1*D*) and decrease of light scattering that affects the excitation spectrum from about 450 up to 550 nm (Naqvi *et al.* 1997) can contribute to decreased Chl *b* and Cars excitation band in Dm samples.

Thus, in our study the isolation of thylakoid membranes from barley leaves affected the pigment composition and the functional state of the PSII complexes only slightly. Despite a disrupted excitation energy transfer from LHCII to PSII antenna core in Dm-solubilized thylakoid membranes, energy transfer from Chl *b* and Cars to emission forms of Chl *a* within LHCII trimers remained effective.

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