A RAPID PROCEDURE FOR ISOLATING THE PHOTOSYSTEM II REACTION CENTERS IN A HIGHLY ENRICHED FORM

The use of a chlorophyll *b*-less mutant of barley

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1. Introduction

Current methods used to isolate chlorophyll a_{II} pigment–protein complexes (chla_{II}–PPC) containing the reaction center of photosystem II in a purified and active form are time consuming, laborious and give low yields [1–5]. Some of the difficulties have been overcome by washing the chloroplasts free of the coupling factor [6] or by using mutant chloroplasts which lack both thylakoid ATPase and chla_I–protein [7]. From this mutant, highly active chla_{II}–PPC has been obtained with a comparatively high chlorophyll recovery, a 4–7-fold enrichment in C 550 and an antenna size of 40–50 chl molecules. Isolation of the reaction centers by this method takes ~20 h, and is one of the shortest procedures hitherto reported.

Here, we describe an isolation method of chla_{II}–PPC, using a chl*b*-less mutant of barley. It has a yield comparable to the former method [7], lasts ⩽6 h, and produces, in addition to chla_{II}–PPC, chla_I pigment–protein complex (chla_I–PPC) as well. Both complexes have an antenna size of ~40 chl molecules.

2. Materials and methods

Seedlings of pea (*Pisum sativum* L. cv. Prevoskhodny) and of a chl*b*-less mutant of barley (*Hordeum vulgare* L. strain 3613 of Gatersleben) were grown in a greenhouse for 3–4 weeks. Leaf tissue of ~30 g fresh wt was harvested and washed with cold-distilled water. All further steps were carried out at 0–5°C.

Chloroplasts were isolated in a medium containing 0.4 M sucrose, 10 mM NaCl, 5 mM sodium ascorbate, 1 mM EDTA, 20 mM Tris–HCl buffer (pH 7.8) [8]. They were disrupted in a hypotonic medium containing 1 mM $MgCl_2$ and 5 mM Tris–HCl (pH 8.0). One litre of this solution contained ~20 mg chl. The undisrupted chloroplasts were separated by centrifugation (at 800 × *g* for 5 min), and the thylakoids from the supernatant were pelleted at 5000 × *g* for 20 min.

The pelleted membranes were suspended in 5 mM Tris–HCl (pH 8.0), 3.3% Triton X-100 at a Triton X-100/chl ratio of 100/1 (w/w). This ratio can be varied between 80–120, depending on the plant material. Solubilization of the thylakoid membranes was carried out in an ice-bath for 1.5–3.0 h. The solubilized material was centrifuged at 3000 × *g* for 15 min to sediment starch grains. Aliquots of the solubilized material containing 2 mg chl were layered on short (3 cm diam. × 4 cm) columns of DEAE-cellulose (0.6–0.8 mequiv./g Reanal, Hungary) pre-equilibrated with 0.05% Triton X-100 in 5 mM Tris–HCl (pH 8.6) a pH preferred for the separation of the chla_{II}–protein particles [9]. The flow rate through the column was 1.5–2.5 ml/min. The columns were washed with the same buffer to remove the solubilized chlorophyll and to decrease [Triton X-100] to 0.05%. The band containing particles enriched in chla_I–protein was eluted by 75 mM NaCl, 0.05%

Abbreviations: chl, chlorophyll; Ph, pheophytin; DPIP, 2,6-dichlorophenol indophenol; chla_{II}–PPC and chla_I–PPC, pigment–protein complexes enriched in photosytem II and photosystem I reaction center, respectively

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Triton X-100 in 5 mM Tris–HCl (pH 8.6). The chla_{II}–PPC band was eluted by 300 mM NaCl, 0.05% Triton X-100 in 5 mM Tris–HCl (pH 8.0) [4,9]. The chromatography was completed in <1 h. The chla_{II}–protein fraction was passed through Sephadex G-25 columns in order to remove the Triton X-100 and NaCl.

Absorbance changes induced by continuous actinic light were measured in a phosphoroscopic photometer as in [10]. Hill activity of the samples was determined according to [4].

The concentration of photosystem II reaction centers in chla_{II}–PPC was estimated on the basis of reversible light-induced absorption changes of pheophytin at 685 nm (ΔA_{685}) which in photosystem II acts as an intermediary electron acceptor between P680 and Q [11,12]. Photooxidation of the primary electron donor, P680, as revealed by the absorbance changes at 680 nm, was also used as a photosystem II activity test [12]. Purity of the preparations in terms of contamination by photosystem I was monitored by light-induced ΔA at 700 nm (ΔA_{700}) as in [4,9].

3. Results and discussion

Fig.1 and 2 show the kinetics and spectra of the light-induced absorbance changes related to the photoreduction of pheophytin and photooxidation of P680 in chla_{II}–PPC isolated from the mutant barley. Both characteristics are similar to those in [11,12] for a preparation of chla_{II}–PPC from normal pea chloroplasts. The preparations were sufficiently stable to allow protracted experimentation. However, after several days at 5°C, the activity of the chla_{II}–PPC decayed significantly, while the activity of the contaminating chla_{I}–PPC remained unchanged. This results in a virtual decrease of the purity of the sample by the proportional increase in contamination by photosystem I.

The Hill-activity (measured by electron transfer from DPC to DPIP) of the chla_{II}–PPC preparation obtained from the mutant was 280 mol DPIP reduced . mol chl^{-1} . h^{-1}, a value similar to that measured in preparations from normal chloroplasts [2–4].

The purity of the preparations and reproducibility of the method is demonstrated in table 1.

An important point of the method described for the isolation of chla_{II}–PPC is the use of a slightly alkaline medium (pH 8.6) during the solubilization of lamellae and the subsequent separation of chla_{II}–PPC

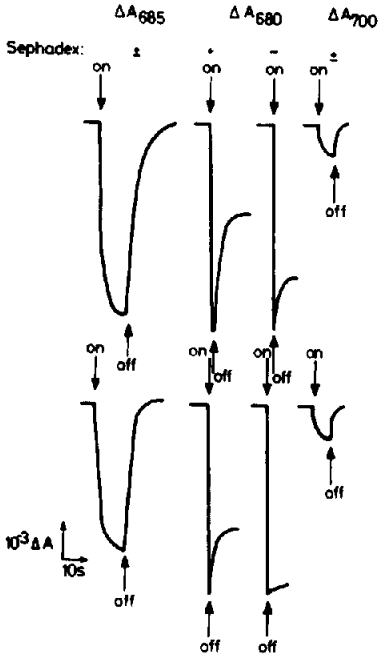

Fig.1. Kinetics of the light-induced absorbance changes of chla_{II}–PPC obtained from chl$b$-less mutant barley: upper, fresh preparation; lower, preparations stored for 3 days at 5°C. The medium contained 20 mM Tris–HCl and 35 mM NaCl; the chlorophyll content was adjusted to 10^{-5} M. Light-path was 1 cm. For measuring ΔA_{685}, the samples were supplemented by 2 mg sodium dithionite/ml and 5 μM methyl viologen. For measuring ΔA_{680}, 1 mM potassium ferricyanide was added to the samples. ΔA_{700} was assayed in the presence of 50 μM sodium ascorbate and 50 μM 2,6-DPIP. Sephadex + or –: after or before treatment with Sephadex G-25.

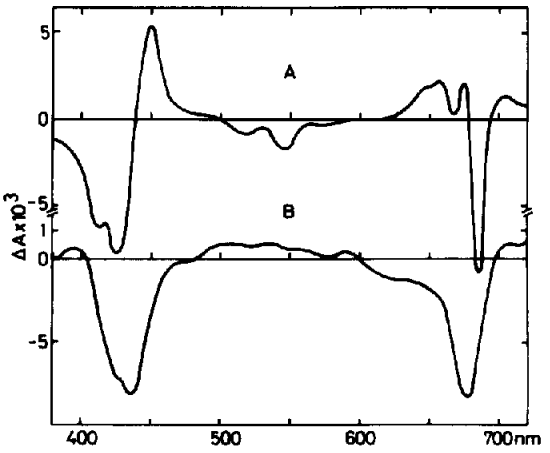

Fig.2. Light-minus-dark difference spectra of chla_{II}–PPC obtained from the chl$b$-less mutant of barley: (A) photoreduction of pheophytin; (B) photooxidation of P680. The reaction mixtures were the same as in fig.1 for measuring ΔA_{685} and ΔA_{680}, respectively.

Table 1
Separation characteristics of chla_{II}–PPC and chla_I–PPC from chloroplasts of normal pea and chl*b*-less, mutant barley seedlings

Material	chl*a* / chl*b*	Ph (10^{-5} M)	P700 (10^{-5} M)	Ph / P700	chl / Ph	chl / P700
Normal pea						
chla_{II}–PPC	5.7	18.7 ± 4.7	1.6 ± 0.3	11.7	53	
chla_I–PPC	3.2	0.8 ± 0.1	6.1 ± 0.6	0.1		163
Mutant barley						
chla_{II}–PPC	–	22.5 ± 3.8	1.5 ± 0.3	15.0	40	
chla_I–PPC	–	7.6 ± 1.2	31.2 ± 0.9	0.2		32

Mean ± SD values from 4 expt. Measuring conditions were the same as in fig.1. The initial chl/P700 value in normal pea chloroplasts was 620 ± 30, and in chl*b*-less chloroplasts of barley 410 ± 25. The amount of Ph was calculated with a difference extinction coefficient of 32 $mM^{-1} \cdot cm^{-1}$ at 685 nm [15], and the amount of P700 with that of 64 $mM^{-1} \cdot cm^{-1}$ at 700 nm [16]

and chla_I–PPC on DEAE-cellulose.

As shown in [9], slightly alkaline conditions are favourable for the binding of chla_I–PPC with the light-harvesting complex, as well as for the loosening of their interaction with the chla_{II}–PPC. This leads to an in vitro association of chla_I–PPC with the light-harvesting complex and to their co-elution by 75 mM NaCl during the isolation of the complexes obtained from normal plants. This can be clearly seen from the high chl/P700 ratio in table 1. The chla_{II}–PPC obtained by this procedure has a small antenna chlorophyll and contains small amounts of photosystem I.

During separation of the complexes on DEAE-cellulose prepared from mutant chloroplasts, an intense green zone of free chlorophyll was observed which was much broader than with normal plants. The main source of the solubilized chlorophyll was probably a pool of loosely bound chlorophyll which is incorporated into the normal light-harvesting complex. Due to the deficiency of the light-harvesting complex in the mutant [13,14], the solubilization of chl*b*-less thylakoids required a shorter time, and the separation of chla_{II}–PPC and chla_I–PPC was more efficient with the mutant than with the normal chloroplasts.

The results show that the use of the chlorophyll *b*-less mutant for the isolation of reaction center complexes has the following advantages:

(i) The chla_{II}–PPC obtained from the mutant is more enriched in photosystem II reaction centers than those from normal chloroplasts;
(ii) In addition to chla_{II}–PPC, the mutant yields highly purified chla_I–PPC.

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