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PREPARATION OF O_2-EVOLVING PHOTOSYSTEM-II SUBCHLOROPLASTS FROM SPINACH *

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An O_2-evolving Photosystem II subchloroplast preparation was obtained from spinach chloroplasts, using low concentrations of digitonin and Triton X-100. The preparation showed an O_2 evolution activity equivalent to 20% of the uncoupled rate of fresh broken chloroplasts, but had no significant Photosystem-I-dependent O_2 uptake activity. The preparation showed a chlorophyll *a/b* ratio of 1.9 and a *P*-700/chlorophyll ratio of 1/2400. Absorption spectra at room temperature and fluorescence emission spectra of chlorophyll at 77 K suggested a significant decrease in Photosystem I antenna chlorophylls in the O_2-evolving Photosystem II preparation.

Oxygen evolution activity of chloroplasts is quite easily lost by detergent treatment and physical disruption of the thylakoid membrane systems that have been used for isolation of PSII reaction center particles. Recently, a highly active O_2-evolving PSII preparation was isolated from membranes of the thermophilic blue-green alga *Phormidium laminosum*, by treatment with lauryldimethylamine oxide followed by a Sepharose 6B column chromatography [1,2]. The O_2-evolving enzyme system of the thermophilic alga seems to be stable enough to withstand the relatively strong detergent treatment and long preparation procedure. In the present paper we report the preparation of PSII subchloroplast fragments with high O_2-evolution activity from spinach chloroplasts. Care was taken to use a combination of rather mild detergents and to shorten the whole isolation procedure.

Spinach (*Spinacia oleracea*, cv. World Best, Yamato Nohen Seed Co., Japan) was grown in the experimental field of our laboratory under natural daylight conditions. Well-developed young leaves were harvested 3–4 weeks after seeding. Broken chloroplasts were prepared by the partially modified method of Lilley and Walker [3]. Deribbed washed leaves (60 g) were ground quickly for 2–3 s in 120 ml of an ice-cooled buffer medium comprising 0.33 M sorbitol/10 mM $Na_4P_2O_7$/4 mM $MgCl_2$ (pH adjusted to 6.5 with HCl) (hereafter referred to as buffer medium) in the presence of 2 mM sodium ascorbate. The homogenate was squeezed through four layers of gauze and then filtered through eight layers of gauze containing two layers of cotton wool. The filtrate was then centrifuged at 8000 × *g* for 5 s and pellet was suspended in the 25-fold diluted buffer medium. The suspension was allowed to stand for 1 min, and then centrifuged at 8000 × *g* for 5 min. The resulting pellet was resuspended in a small volume of the non-diluted buffer medium. The broken

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Abbreviations: PS, Photosystem; Chl, chlorophyll; DCIP, 2,6-dichloroindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

chloroplasts (Chl concentration adjusted to 0.5 mg/ml) were incubated with digitonin (0.25%) and the suspension was stirred for 5 min in the ice bath. After the digitonin treatment, the suspension was diluted 10-fold with buffer medium and centrifuged at 10 000 × *g* for 30 min. The pellet was resuspended in buffer medium (Chl concentration, 0.5 mg/ml) and 0.2% Triton X-100 was added. The suspension was stirred again for 5 min in the ice bath and then diluted 10-fold with buffer medium. Centrifugation of the suspension at 12 000 × *g* for 30 min resulted in the separation of pale-green supernatant from the dark-green pellet. After resuspension in a small volume of buffer medium, the pellet was used as a PSII subchloroplast preparation.

O_2 evolution and PSI-dependent O_2 uptake were measured with a Rank oxygen electrode. The assay medium for O_2 evolution (2 ml) contained 0.5 mM *p*-benzoquinone and 5 mM NH_4Cl in buffer medium. O_2 uptake was assayed in the presence of 0.1 mM methyl viologen, 50 μM DCIP, 0.5 mM sodium ascorbate, 0.5 mM sodium azide and 10 μM DCMU in buffer medium. Broken chloroplasts and subchloroplast preparations were used at a concentration of 10 μg Chl/ml. Illumination was provided by a 650 W projector with a Toshiba R-62 filter. The temperature of the reaction mixture was adjusted to 20°C by circulating thermostatically controlled water around the reaction vessel. Absorption spectra of the preparations were measured with a Shimadzu UV-240 spectrophotometer. The *P*-700 content of the preparations was determined by ascorbate-minus-ferricyanide difference spectra using a value of 64 $mM^{-1} \cdot cm^{-1}$ as ϵ_{700nm} for *P*-700 [4]. Fluorescence emission spectra at 77 K were measured using a Hitachi MPF-2A fluorescence spectrophotometer. Excitation wavelength was 430 nm and the half-bandwidth of the emission monochromator was 3 nm. Chemicals used were of the analytical grade. *p*-Benzoquinone was purified by sublimation. Digitonin was recrystallized from alcoholic solution.

Even mild detergent treatment of chloroplasts caused a rather significant solubilization of Chl or

TABLE I

CHLOROPHYL *a/b* RATIO, *P*-700/CHLOROPHYL RATIO AND ESTIMATED YIELD OF SUBCHLOROPLAST PREPARATIONS AFTER DETERGENT TREATMENT OF CHLOROPLASTS

Chl *a/b* ratio is the average of five measurements ± S.D. *P*-700/Chl ratio is the average of three measurements. The yield of the preparations is expressed in the relative amount of chlorophyll recovered in the pellet obtained by centrifugation at 10 000 × *g* for digitonin subchloroplasts or at 12 000 × *g* for digitonin/Triton subchloroplasts after the detergent treatment.

preparation	Chl *a/b*	*P*-700/Chl	Chl recovered (%)
Broken chloroplasts	3.4 ± 0.4	1/440	100
Digitonin subchloroplasts	2.6 ± 0.3	1/770	53
Digitonin/Triton subchloroplasts	1.9 ± 0.4	1/2400	29

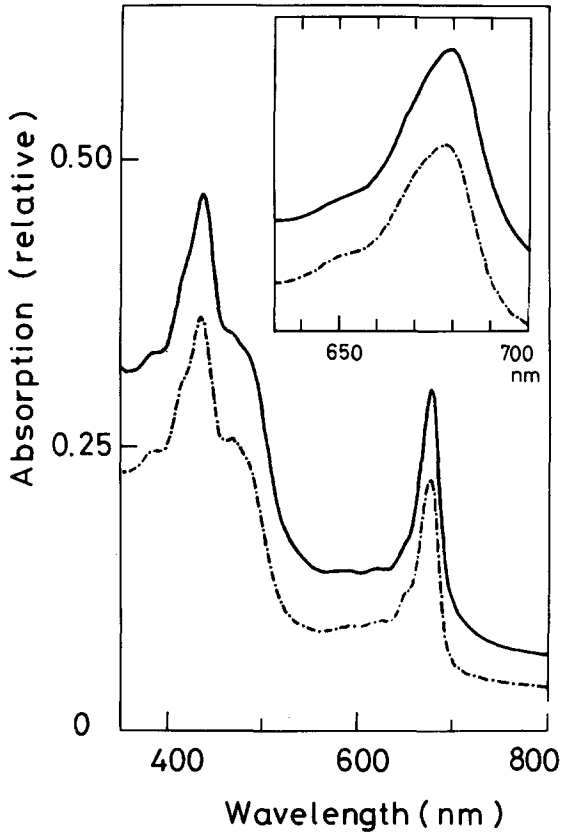

Fig. 1. Absorption spectra of broken chloroplasts and PSII subchloroplast preparation. Inset shows the red absorption bands on expanded wavelength scale. Solid line, broken chloroplasts; broken line, PSII preparation.

Chl-protein complexes from thylakoid membranes (Table I). A 5-min treatment of chloroplasts with 0.25% digitonin removed more than 30% of chlorophyll from thylakoids and the succeeding 5-min treatment with 0.2% Triton X-100 resulted in a further solubilization of about 50% of chlorophyll in the digitonin-treated preparation.

The Chl *a*/*b* ratio decreased to 1.9 as preparation steps for PSII subchloroplasts proceeded. The increase in Chl *b* in the PSII preparation was also apparent when the absorption spectra of broken chloroplast and digitonin/Triton-treated subchloroplast preparations were compared; in the PS-II preparation, shoulder of Chl *b* absorption at 470 nm and 650 nm became prominent (Fig. 1). Furthermore, the red absorption peak of the broken chloroplasts was shifted by 2–3 nm toward shorter wavelength after the digitonin/Triton-treatment of chloroplasts (Fig. 1, inset), suggesting the enrichment of shorter-wavelength forms of Chl in the PSII preparation. The ratio of *P*-700 to Chl was 1/2400 in the PSII preparation. The value was more than 5-times smaller than that in broken chloroplasts.

TABLE II

ACTIVITY OF O_2 EVOLUTION AND O_2 UPTAKE IN BROKEN CHLOROPLAST AND SUBCHLOROPLAST PREPARATIONS AFTER DETERGENT TREATMENT

Preparation and conditions	O_2 evolution (μmol O_2/mg Chl per h)	O_2 uptake [a] (μmol O_2/mg Chl per h)
Broken chloroplasts	95	210
Broken chloroplasts + NH_4Cl [b]	320	
Broken chloroplasts + NH_4Cl + DCMU [c]	0	
Digitonin subchloroplasts + NH_4Cl	430	260
Digitonin/Triton subchloroplasts + NH_4Cl	65	15
Digitonin/Triton subchloroplasts + NH_4Cl + DCMU	0	

[a] Measured without NH_4Cl.
[b] NH_4Cl, 5 mM.
[c] DCMU, 10 μM.

The PSII preparation showed an O_2 evolution activity equivalent to 20% of the uncoupled rate in the fresh broken chloroplasts, but had only a very low PSI-dependent activity of O_2 uptake (Table II). The O_2 uptake activity of the PSII preparation was not affected significantly by the addition of plastocyanin to the assay medium (only about 50% increase in the residual activity was observed in the presence of 1 μM plastocyanin). The O_2 evolution activity was inhibited by DCMU, which indicated that the O_2 evolution was of the PSII nature. Treatment of chloroplasts with digitonin alone significantly increased the specific activity of O_2 evolution (Table II), but had no marked effect in reducing the PSI activity. Digitonin has been used for separation of stroma lamellae from PSII-enriched grana lamellae [5]. In our experiments, the broken chloroplasts were suspended in the medium containing 4 mM $MgCl_2$ to induce stacking of thylakoids. As exclusion of most of the PSI complexes from the grana partitions has been observed [6,7], the stacking may be a prerequisite condition for the successful digitonin treatment. Triton X-100 was found to be a more effective detergent in removing PSI from the thylakoid membranes [8]. However, Triton X-100 treatment itself inactivated O_2 evolution significantly in parallel with the solubilization of PSI from the thylakoid membranes, and thus the combined detergent treatment (digitonin and Triton X-100, see also Ref. 9) was the most appropriate method of those tried. As the prolonged incubation of chloroplasts with either digitonin or Triton X-100 induced inactivation of O_2 evolution, we removed the detergents from the membrane preparations as quickly as possible by dilution and centrifugation steps after each detergent treatment.

Fluorescence emission spectra of the PSII preparation showed a large decrease in the intensity of emission band at 735 nm compared with the broken chloroplasts, although the preparation still contained a small amount of PSI antenna Chl that is responsible for 735 nm fluorescence emission band (Fig. 2). The ratio of F_{735} (intensity of fluorescence at 735 nm) to F_{685} (that at 685 nm) in the PSII preparation decreased by a factor of more than four compared with the broken chloroplast.

Our whole preparation procedure for the O_2-evolving PSII preparation needed only 3 h and the yield was quite high (Table I). The detergent treatment of

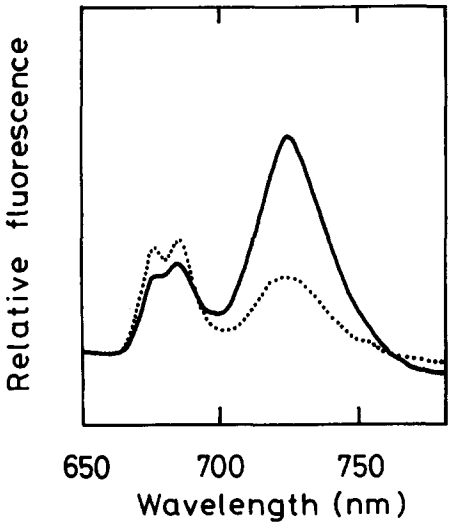

Fig. 2. Fluorescence emission spectra of broken chloroplast (solid line) and PSII subchloroplast preparations (dotted line) at the liquid N_2 temperature.

chloroplasts gave variable results depending on the starting materials. So, it is essential to use fresh spinach leaves grown under well-controlled conditions. Study is being undertaken for further purification and characterization of the O_2-evolving PSII preparation.

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