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A RAPID, HIGH-YIELD METHOD FOR THE PURIFICATION OF THE WATER-SOLUBLE 33 KILODALTON PROTEIN OF SPINACH THYLAKOIDS

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A new purification procedure for the water-soluble 33 kDa protein of Photosystem II is presented. The method is based on the selective release of the 33 kDa protein at slightly elevated temperatures and involves a mininum of purification steps. Starting with spinach leaves, the pure protein may be obtained in about 4 h, with a yield usually higher than 60 %.

Several studies have indicated the involvement of a water-soluble 33 kDa protein in photosynthetic oxygen evolution (1-7). It is released from PS II preparations by high concentrations of Tris, urea or divalent cations, together with two components with molecular masses 16 and 24 kDa (2-7). The 33 kDa protein was first characterized by Kuwabara and Murata, who isolated it from PS II particles (8), or from acetone-extracted thylakoids (9). Here we present a new purification procedure for the protein, which avoids the use of organic solvents and time-consuming chromatographic steps.

MATERIALS AND METHODS

Spinach thylakoids were prepared as in (10) and suspended in 50 mM Hepes-NaOH (pH 7.5) and 10 mM NaCl (Hepes buffer). The thylakoids were mixed with 10 % (w/v) deoxycholate giving final concentrations of 5 mg chl/ml and 2 % (w/v) deoxycholate, and incubated on ice for 15 min. After centrifugation at 40 000 x g for 1.5 h, the membrane fraction was suspended in Hepes buffer and again centrifuged at 40 000 x g for 30 min to remove the detergent. The deoxycholate-extracted thylakoids were suspended in Hepes buffer at 4 mg chl/ml and incubated at 55°C for 5 min. This treatment releases the 33 kDa protein specifically, and

Abbreviations: chl: chlorophyll; Hepes: 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; PS II: photosystem II; SDS: sodium dodecyl sulfate; Tris: 2-amino-2-hydroxymethylpropane-1,3-diol

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after centrifugation at 40 000 x g for 30 min, the supernatant was used as a preparation of the 33 kDa protein. However, before characterizations such as amino acid analysis, the protein was dialyzed first against 0.1 M NaCl and 2 mM NH_4Cl and then against 2 mM NH_4Cl and freeze-dried.

SDS-polyacrylamide gel electrophoresis was performed in the buffer system of Laemmli, using 10-20 % gradient gels. The gels were stained with Coomassie Brilliant Blue R-250. Densitometric analyses of the gels were made at 560 nm with a Gilford 240 spectrophotometer equipped with a gel scanning attachment.

The isoelectric point was determined by flat bed electrofocusing, using Ampholine pH 3.5-10 (LKB, Sweden).

The molecular weight was determined by gel filtration on Sephacryl S-200 (Pharmacia Fine Chemicals, Sweden). The column was eluted with 50 mM sodium phosphate (pH 7.0) and 10 mM NaCl. Bovine serum albumin, ovalbumin and myoglobin were used as molecular mass standards.

Optical spectra were recorded on a Beckman Acta MIV double beam recording spectrophotometer.

Amino acid analyses were performed after 20 and 70 h hydrolysis in 6 M HCl at 110°C, using a Beckman 120 B amino acid analyzer.

RESULTS AND DISCUSSION

Preparation of the 33 kDa protein. The deoxycholate extraction removes several extrinsic, water-soluble proteins, among them the 16 and 24 kDa polypeptides, plastocyanin, ribulose-1,5-bisphosphate carboxylase and the coupling factor CF_1 of the chloroplast ATPase, in addition to some intrinsic proteins such as the cytochrome *bf* complex (11). A mild heat treatment of the deoxycholate-extracted membranes results in a specific release of the 33 kDa protein (Fig. 1). After centrifugation, the supernatant can be used as a virtually pure preparation of the protein. In some preparations, a 37 kDa polypeptide, probably the NADP reductase, can be detected by electrophoresis. This impurity usually corresponds to a few per cent of the total protein content, as estimated from Coomassie Blue staining of gels. Since no chromatographic steps are needed, the purification procedure is very rapid, requiring only about 4 h. The yield of the method is very high. Approximately 10 % of the protein is lost in the deoxycholate extraction, and the heat treatment usually releases more than 70 % of the remaining 33 kDa protein from the membranes. Thus, more than 60 % of the initial chloroplast content of the 33 kDa protein is recovered in pure form.

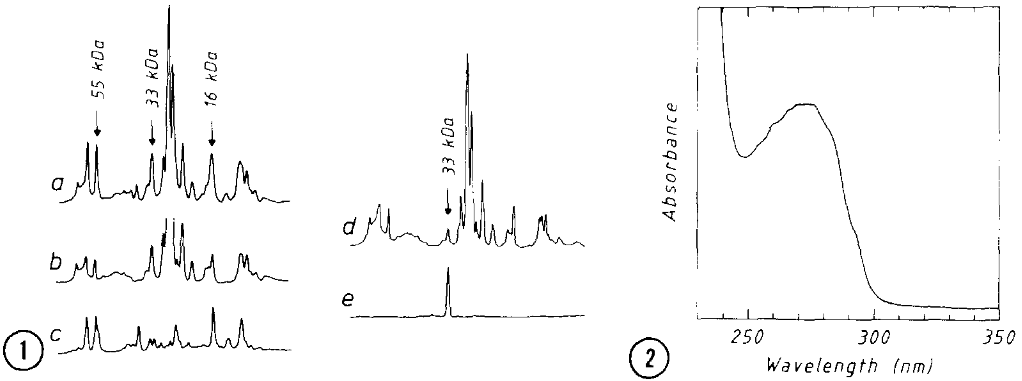

FIG. 1. Densitograms of SDS polyacrylamide gels. a: Thylakoids; b: Deoxycholate-extracted thylakoids; c: Supernatant from deoxycholate extraction; d,e: Membrane fraction and supernatant after heat treatment of deoxycholate-extracted thylakoids (sample e in a concentration corresponding to twice the concentration of sample d).

FIG. 2. Optical spectrum of the 33 kDa protein.

Instead of the heat treatment, an incubation with 0.8 M Tris (pH 8.4) can be used. This treatment also releases the 33 kDa protein, but results in a less pure preparation, only 90-95 % pure (not shown). In addition to the 33 kDa polypeptide, the Tris extract preparation also contains the same 37 kDa polypeptide and small amounts of the 16 and 14 kDa polypeptides which have not been removed by deoxycholate.

Characterization. The isoelectric point of the protein is 5.2. The molecular mass determined by gel filtration is 31 kDa, close to the value of 33 obtained in SDS electophoresis, which shows that the protein is solubilized as a monomer. The ultraviolet spectrum (Fig. 2) shows a peak in the 280 nm region, typical of aromatic amino acids. The protein has no absorption band in the visible region. The amino acid composition (Table 1) shows that the protein is hydrophilic. The polarity index calculated from the amino acid composition (12) is 47 %. The histidine content is very low, corresponding to 0-1 His per protein molecule. This histidine content may result from a contamination, since the preparation used for the amino acid analysis contained approximately 2 % of the 37 kDa polypeptide.

All these characterizations are in good agreement with the results of Kuwabara and Murata (9). Thus, the protein described in this paper is

TABLE 1. Amino acid composition (mol %)
Average of 20 and 70 h hydrolysis

Lys	8.5	Ala	6.9
His	0.26	Cys	0.97[b]
Arg	2.7	Val	7.7[c]
Asp	8.6	Met	0.84
Thr	7.3[a]	Ile	3.7[c]
Ser	7.4[a]	Leu	7.4
Glu	12.0	Tyr	3.3
Pro	5.8	Phe	5.4
Gly	11.1	Trp	ND[d]

[a]Extrapolation to zero hydrolysis time
[b]20 h hydrolysis of performic acid oxidized sample
[c]70 h hydrolysis
[d]Not determined

identical to their 33 kDa protein. The simplicity and the high yield of the preparation method may facilitate further studies of the structure of the 33 kDa protein and its involvement in oxygen evolution and PS II electron transport.

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