Improved purification and N-terminal sequence of the 33-kDa protein in spinach PS II

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Received 24 October 1985

Significant improvement was attained in the purification of a peripheral 33-kDa protein from spinach chloroplasts by the use of butanol/water phase partitioning. Over 30% of the 33-kDa protein in the chloroplasts was isolated in a short time. The amino acid sequence for the first 37 residues from the N-terminus was determined by the automated sequencing of the purified protein.

33-kDa protein *Amino acid sequence* *Oxygen evolution* *Photosystem II* *Photosynthesis*

1. INTRODUCTION

A peripheral protein of the thylakoid membrane, of 33-kDa protein, is one of the essential components in O_2 evolution in chloroplasts [1]. This protein is associated with PS II and seems to play a crucial role in keeping catalytic manganese in situ in the O_2-evolution enzyme complex. Removal of the 33-kDa protein from the O_2-evolving PS II particles allows release of manganese atoms from the membranes and results in inhibition of O_2 evolution [1]. Under certain conditions, the 33-kDa protein can be isolated with bound manganese, which suggests the close interaction of manganese and the 33-kDa protein [2,3].

The isolation of the 33-kDa protein has been reported and the molecular properties of the protein partially revealed [4,5]. The protein is an acidic protein with p*I* of 5.1 and analysis of the amino acid composition showed its hydrophilic nature. The 33-kDa protein is rich in lysine and is easily stained by Coomassie blue in SDS-PAGE gels probably due to the high content of positively charged amino acid residues [6]. These characteristics of the 33-kDa protein contrast with those of the herbicide-binding protein that has almost the same molecular mass as the 33-kDa protein but is hydrophobic and contains no lysine [7,8].

Here, we describe an improved method for purification of the 33-kDa protein from spinach chloroplasts, which might be applicable to the isolation of the similar protein from other plants and algae. With the purified 33-kDa protein we show the N-terminal sequence of amino acids.

Abbreviations: PS, photosystem; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; Mes, 4-morpholine-ethanesulfonic acid; CF_1, coupling factor of chloroplast; FNR, ferredoxin-NADP oxidoreductase

2. MATERIALS AND METHODS

Broken chloroplasts were prepared from 2 kg spinach leaves as described [9]. The chloroplasts were suspended in a solution containing 0.33 M sorbitol, 70 mM Mes and 120 mM NaCl (pH 6.5) (solution A) at a chlorophyll concentration of about 1 mg/ml. The chloroplast suspension was sonicated at 60 W for 30 s in the ice bath. The suspension was then centrifuged at $12000 \times g$ for 12 min at 4°C, and the heavy thylakoid fragments were obtained as a pellet. The pellet, resuspended in solution A, was incubated with Triton X-100 at

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00145793/86/$3.50

a Triton/chlorophyll ratio of 25 (w/w) for 1–2 min with stirring at 4°C. The suspension was centrifuged at 3000 × *g* for 5 min to remove the Triton-resistant thylakoid fraction as a pellet, and the supernatant was centrifuged at 35 000 × *g* for 20 min at 4°C. A PS II-enriched fraction (PS II particles) obtained as a pellet was suspended in solution A.

For NaCl treatment of the PS II particles, the PS II particles were incubated for 30 min on ice with a solution containing 1 M NaCl added to solution A at about 0.1 mg chlorophyll/ml. The suspension was centrifuged at 35 000 × *g* for 30 min at 4°C and the resulting pellet washed once with solution A by repeating centrifugation. The NaCl-washed PS II particles were then subjected to phase partitioning with *n*-butanol as in [3]. The NaCl-washed PS II particles (700 ml) containing about 0.5 mg chlorophyll/ml were mixed with an equal volume of cold *n*-butanol, and the mixture shaken vigorously for 30 s. The mixture was then centrifuged at 3000 × *g* for 5 min and the upper green-colored butanol phase discarded. The lower aqueous phase was carefully collected and applied to a DEAE-Sepharose column (70 × 25 mm) equilibrated with 50 mM Mes (pH 6.5). After washing the column with 5 vols of 50 mM Mes (pH 6.5) to remove the butanol, the 33-kDa protein was eluted by a 0–0.5 M NaCl gradient. The elution of the protein was monitored by a Gilson UV monitor. The 33-kDa protein fraction was desalted and concentrated with an Amicon ultrafiltration cell with a UM10 membrane. Finally the protein was lyophilized and stored at −80°C before use.

SDS-PAGE was carried out with a 10% polyacrylamide gel as described [5]. Amino acid composition of the 33-kDa protein was determined on a Durrum D-500 amino acid analyser according to the manufacturer's instructions. The N-terminal amino acid sequence of the protein was determined by a Beckman model 890C sequencer according to [10].

3. RESULTS AND DISCUSSION

The peripheral 33-kDa protein is associated with the PS II particles prepared by detergent treatment of spinach chloroplasts. Here, the PS II particles were obtained by brief sonication and treatment with Triton X-100 of broken chloroplasts. Sonication of chloroplasts in the presence of an appropriate concentration of salt (120 mM NaCl in this study) and the subsequent centrifugation yielded a grana fraction enriched in PS II. The grana fraction was then treated with Triton X-100 to remove PS I complex and the other membrane components not related to PS II from the membranes. It was not necessary to remove completely these components from the membranes, because the peripheral proteins other than the 33-kDa protein were released almost completely by washing the membranes with 1 M NaCl prior to phase partioning. The hydrophobic membrane components were separated from the 33-kDa protein phase partitioning with butanol as described below. To increase the yield of the 33-kDa protein, it is preferable to obtain a large amount (500 mg chlorophyll or more) of the PS II-enriched fraction in a reasonably pure state.

Washing the PS II particles with 1 M NaCl has been shown to be effective in removing the PS II-associated 24- and 18-kDa proteins from the membranes [1,11]. The NaCl treatment was also a necessary step to remove a small amount of CF_1 and FNR from the PS II particles. FNR of about 35-kDa has almost the same p*I* as the 33-kDa protein and was difficult to separate from the 33-kDa protein by molecular sieve or ion-exchange chromatography. Hydroxyl apatite column chromatography was applied previously to separate these 2 proteins from each other [12], but we found that prewashing of the PS II particles with 1 M NaCl was a rather quick and efficient way to remove the contaminating FNR from the PS II particles. Thus, the 33-kDa protein was the only peripheral protein present in the NaCl-washed PS II particles. From 2 kg spinach leaves, we usually obtained broken chloroplasts, the PS II particles and NaCl-treated PS II particles equivalent to 1500, 500 and 350 mg chlorophyll, respectively.

Phase partitioning with *n*-butanol is a simple way to separate the hydrophilic from hydrophobic proteins in the membranes, and was first applied successfully to erythrocyte membranes [13]. The 33-kDa protein in the NaCl-washed PS II particles was quickly isolated by phase partitioning with *n*-butanol, and applied to the DEAE-Sepharose column (fig.1). SDS-PAGE of the protein obtained after column chromatography showed a single band at 33-kDa (fig.2). With this procedure we ob-

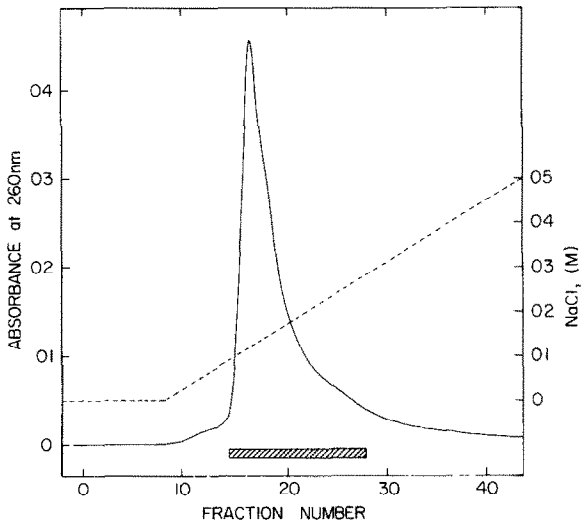

Fig.1. Elution profile of the 33-kDa protein in DEAE-Sepharose column chromatography. The 33-kDa protein was eluted by 0–0.5 M NaCl gradient. The hatched fractions were collected.

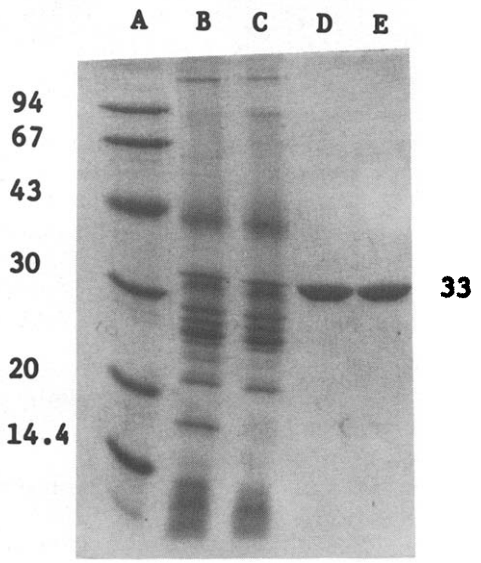

Fig.2. A slab gel of SDS-PAGE showing the purification of the 33-kDa protein. Lanes: A, marker proteins; B, PS II particles (5 μg chlorophyll); C, PS II particles washed with 1 M NaCl (5 μg chlorophyll); D, E, the 33-kDa protein obtained after DEAE-Sepharose column chromatography.

Table 1

Amino acid composition of the 33-kDa protein

Amino acid	mol amino acid/mol 33-kDa protein
Aspartic acid	26.1
Threonine	22.7
Serine	20.1
Glutamic acid	38.4
Proline	17.5
Glycine	36.3
Alanine	17.4
Half-cystine	N.D.
Valine	23.9
Methionine	1.7
Isoleucine	9.4
Leucine	21.3
Tyrosine	8.7
Phenylalanine	15.9
Histidine	0.0
Lysine	29.1
Arginine	7.7
Tryptophan	N.D.
Polarity index	49%

The protein was hydrolyzed for 24 h at 110°C with 6 N HCl. N.D., not determined. As the amounts of half-cystine and tryptophan were not taken account of in the calculation of mol amino acid/mol 33-kDa protein, the real values of mol amino acids are expected to be a little lower than those presented here

tained about 43 mg purified 33-kDa protein from 2 kg spinach leaves. Assuming that the antenna size of the broken chloroplasts is about 400 chlorophylls, and judging from the data that one molecule of the 33-kDa protein is present per PS II reaction center [14], we suggest that more than 30% of the 33-kDa protein in the broken chloroplasts was isolated by this method. It takes less than a day to purify ~40 mg of the 33-kDa protein by this procedure.

Amino acid analysis extends the identification of this protein with the 33-kDa protein of the O_2-evolution enzyme complex (table 1). The numbers of polar and non-polar amino acids (table 1) appearing in this protein are almost equal, reflecting the polarity index of the protein of ~50% [5]. The 33-kDa protein is associated with the PS II reaction center complex, and hydrogen bonds may be involved in the interaction of these 2 components

Table 2

N-terminal amino acid sequence of the 33-kDa protein

1				5				10	
NH_2-Glu-	Gly-	Lys-	Arg-	Leu-	Thr-	Tyr-	Asp-	Glu-	
				15				20	
-Ilu-	Asn-	Ser-	Lys-	Thr-	Tyr-	Leu-	Glu-	Val-	Lys-
				25				30	
-Gly-	Thr-	Gly-	Thr-	Ala-	(Asn)-	Glu-	X-	Pro-	(Thr)-
				35					
-Val-	(Glu)-	Gly-	Gly-	Val-	(Lys)-	(Ser)-	....		-COOH

X, not identified. ( ), not confirmed

[15]. The 33-kDa protein also interacts with the 24-kDa protein through electrostatic forces [15]. Thus the 33-kDa protein is expected to have some surface domains favorable to these protein-protein interactions. The 33-kDa protein may have a binding site of manganese near its surface. The binding of manganese to the 33-kDa protein seems to be dependent on the redox state of manganese, and it was suggested that some conformational change in the O_2-evolution enzyme complex is involved in the binding of the highly oxidized manganese to the 33-kDa protein [3].

A partial amino acid sequence was determined with the purified 33-kDa protein (table 2). The N-terminal residue of the protein is glutamic acid and the next 36 residues were identified. The high fidelity of the automated Edman degradation run is the best proof of the purity of this protein. Further sequencing is required for the analysis of the possible interaction of the 33-kDa protein and manganese.

ACKNOWLEDGEMENTS

The authors appreciate the generous help of Cathy Overholt and Scott Buckle in the determination of the amino acid sequence and amino acid composition of the 33-kDa protein.

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