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AN IMPROVED PURIFICATION METHOD AND A FURTHER CHARACTERIZATION OF THE 33-KILODALTON PROTEIN OF SPINACH CHLOROPLASTS

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The 33-kDa protein was purified in a high yield from thylakoid membranes of spinach chloroplasts. The extinction coefficient and $A^{1\%}_{1cm}$ value at 276 nm of the protein were 22000 $M^{-1} \cdot cm^{-1}$ and 6.8, respectively. The 33-kDa protein and a polypeptide appearing at 32 kDa in the SDS-polyacrylamide gel electrophoresis of thylakoid membranes were compared by peptide mapping after limited proteolysis. This indicates that the 32-kDa band is entirely due to the 33-kDa protein. The molar ratio of chlorophyll to the 33-kDa protein in the chloroplasts was estimated to be 300. This suggests that one photosynthetic unit possesses one or two molecules of the 33-kDa protein.

Introduction

Membranous polypeptides of chloroplasts having molecular masses of 30 to 35 kDa have become a subject of intense investigation in the biochemical research of photosynthesis [1–7]. We have isolated a protein having a molecular mass of 33 kDa from the PS II membrane fragments of spinach chloroplasts, and described its chemical characteristics [1]: The 33-kDa protein is acidic (pI = 5.2) and contains a high proportion of hydrophilic amino acids (polarity index = 49%). It is found in PS II particles but not in PS I particles.

In the present paper, we will describe an efficient method to prepare the 33-kDa protein from spinach chloroplasts, and its further chemical characterization.

Abbreviations: PS, photosystem; Mes, 4-morpholineethanesulfonic acid

Materials and Methods

Chloroplasts were prepared from spinach leaves according to the method described in the previous paper [1] except that the preparation medium contained 0.4 M sucrose, 0.01 M NaCl, 0.05 M Tris-HCl, pH 7.8. The chloroplasts (equivalent to 300 mg chlorophyll) were suspended in 800 ml distilled water and collected by centrifugation at 15000 × g for 15 min. The thylakoid membranes thus prepared was resuspended in 800 ml distilled water, and to the suspension 3200 ml cold acetone (−20°C) were added with stirring. The acetone powder was collected by centrifuging the suspension at 3500 × g for 15 min, and was suspended in 100 ml of 1 mM sodium phosphate buffer, pH 6.8. After stirring in a cold room for 1 h, the suspension was centrifuged at 3500 × g for 15 min. The pellet was again suspended in 100 ml of the above-mentioned buffer and the suspension was centrifuged as above. The supernatant solutions of the phosphate buffer were combined and centrifuged at 220000 × g for 1 h to remove precipitates.

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The resultant supernatant is termed the crude extract.

All procedures of column chromatography with DEAE-Sepharose CL-6B and hydroxyapatite were performed in a cold room. The elution of proteins from the chromatographic columns was monitored at 280 nm (Atto, Mini UV monitor I). The elution of the 33-kDa protein was detected by SDS-polyacrylamide gel electrophoresis.

The SDS-polyacrylamide gel electrophoresis was carried out in a buffer system according to the method of Laemmli [8] except that 2-mercaptoethanol was omitted from the solvent. Electrophoresis was performed with a slab gel apparatus (Atto, SJ-1060). The thickness of the gel was 0.1 cm. The stacking gel contained 5% polyacrylamide, and the separation gel contained a linear gradient of polyacrylamide concentration from 10 to 15% with a gradient of sucrose concentration from 5 to 20%. The gel plates were stained by Coomassie brilliant blue R-250 as described previously [1].

The peptide mapping was performed by gel electrophoresis after limited proteolysis according to the method of Cleveland et al. [9]. A narrow zone of polyacrylamide gel containing the 32 kDa band was excised and soaked in 0.1% SDS and 0.125 M Tris-HCl, pH 7.8, for 30 min and then homogenized with a microhomogenizer in 0.2 ml of 0.5% SDS, 10% glycerol and 0.125 M Tris-HCl, pH 7.8. The homogenate was centrifuged at 6500 $\times g$ for 5 min. The supernatant was divided into 0.04-ml aliquots, and to each aliquot 0.01 ml of 10% glycerol and 0.125 M Tris-HCl, pH 6.8, containing 1 μg protease of *Staphylococcus aureus* strain V-8, was added. The proteolytic digestion was carried out at 37°C for designated periods, and stopped by treating the reaction mixture at 100°C for 1 min in the presence of 2% SDS and 3 mM dithiothreitol. Then, the free sulfhydryl groups were blocked by treating the mixture with 30 mM iodoacetamide at room temperature for 75 min. Electrophoresis of the digested sample was performed as described above. The gel plates were stained with $AgNO_3$ according to the method of Switzer et al. [10].

The molar extinction coefficient and $A^{1\%}_{1cm}$ value at 276 nm were determined from the dry weight, the molecular mass (33 kDa) and the light absorbance in 0.15 M NaCl and 0.025 M Mes-NaOH, pH 6.6. The dry weight of the 33-kDa protein was determined after it was dried in an oven at 105°C for 5 days. Absorption spectra were measured with a recording spectrophotometer (Hitachi 340).

The content of 33-kDa protein in the thylakoid membranes and the fractions at each purification step were estimated from the staining intensity of the band at 32 kDa on the polyacrylamide slab gel. The gel plates were stained with Fast green FCF and destained in 7% acetic acid as described by Gorovsky et al. [11]. Densitograms were measured by tracing the absorbance at 590 nm with 725 nm as a reference beam [1]. In a range from 0 to 4 μg of the 33-kDa protein applied to a slot of 1 cm width, the staining intensity was linearly related to the amount of 33-kDa protein.

The molecular mass and the isoelectric point of the 33-kDa protein were measured, and the discontinuous electrophoresis of the protein was performed as described in the previous paper [1]. Chlorophyll concentration was determined according to the method of Arnon [12].

Spinach was purchased from a local market. DEAE-Sepharose CL-6B was obtained from Pharmacia (Uppsala) and hydroxyapatite from Seikagaku Kogyo (Tokyo). *S. aureus* V-8 strain protease was obtained from Miles (Elkhart). Proteins of molecular mass standard were purchased from Boehringer (Mannheim). Fast green FCF was obtained from Chroma (Stuttgart).

Results

Improved purification method for the 33-kDa protein

From the acetone powder of the thylakoid membranes of spinach chloroplasts, the 33-kDa protein was extracted with 1 mM sodium phosphate buffer, pH 6.8. The SDS-polyacrylamide gel electrophoresis of the crude extract revealed about 20 bands of polypeptides, among which the 33-kDa protein, the subunits of chloroplast coupling factor 1 (CF_1), and unknown polypeptides appearing at 36, 22, 14 and 10 kDa were distinct (Fig. 1c). When the chloroplasts, which contained 300 mg chlorophyll and 37 mg of the 33-kDa protein, were used as starting material, the crude extract contained 12 mg of the 33-kDa protein. Therefore,

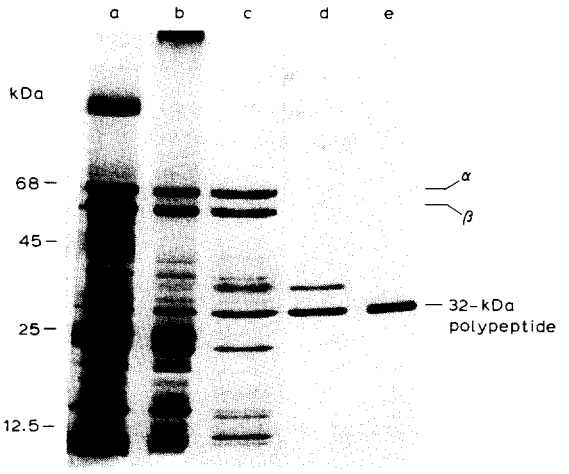

Fig. 1. SDS-polyacrylamide gel electrophoresis of the thylakoid membranes and 33-kDa protein. (a) Thylakoid membranes, (b) acetone powder of thylakoid membranes (c) crude extract, (d) DEAE-Sepharose fraction, (e) 33-kDa protein after column chromatography with hydroxyapatite. α and β represent the α and β subunits of CF_1. The molecular mass range is indicated in kDa. Proteins used as molecular mass (in parentheses) standards were bovine serum albumin (68000), ovalbumin (45000), chymotrypsinogen A (25000) and cytochrome *c* (12500).

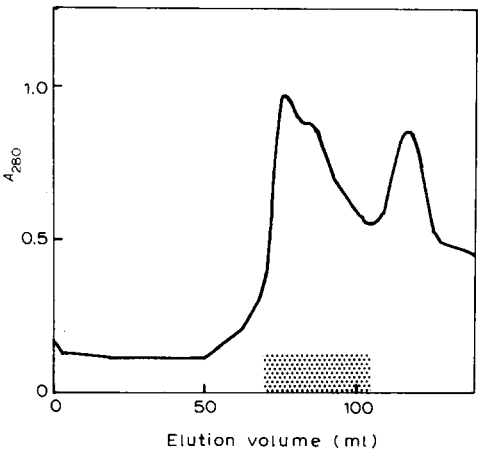

Fig. 2. Column chromatogram with DEAE-Sepharose CL-6B of the crude extract. The column size was 2.6 cm diameter and 15 cm height. The effluent contained 0.15 M NaCl and 0.02 M sodium phosphate buffer, pH 6.5. The flow rate was 40 cm/h. Fractions at elution volumes from 65 to 105 ml indicated by dots were collected as the DEAE-Sepharose fraction.

about 30% of the 33-kDa protein in the thylakoid membranes was recovered in the crude extract.

The crude extract was subjected to a column of DEAE-Sepharose CL-6B which had been equilibrated with 0.03 M NaCl and 0.02 M sodium phosphate buffer, pH 6.7. The column was washed with 0.05 M NaCl and 0.02 M sodium phosphate buffer, pH 6.6. The 33-kDa protein was eluted with 0.15 M NaCl and 0.02 M sodium phosphate buffer, pH 6.5 (Fig. 2). The DEAE-Sepharose fraction contained the 33-kDa protein amounting to 7 mg, a polypeptide having a molecular mass of 36 kDa and several minor components (Fig. 1d). The subunits of chloroplast coupling factor 1 and the unknown components at 22, 14 and 10 kDa were removed by this step.

The DEAE-Sepharose fraction was subjected to a column of hydroxyapatite, which had been equilibrated with 0.02 M sodium phosphate buffer, pH 6.7. Minor components were washed out with 0.125 M sodium phosphate buffer, pH 6.6. The 33-kDa protein was eluted by 0.125 M sodium phosphate buffer with a pH gradient from 6.6 to 6.9 (Fig. 3). The polypeptide at 36 kDa in the SDS-polyacrylamide gel electrophoresis remained adsorbed at the top of the column, and was eluted with 0.4 M sodium phosphate buffer, pH 6.9. It seemed identical with ferredoxin-NADP oxidoreductase [13] with respect to the absorption spectrum and molecular mass. The 33-kDa protein

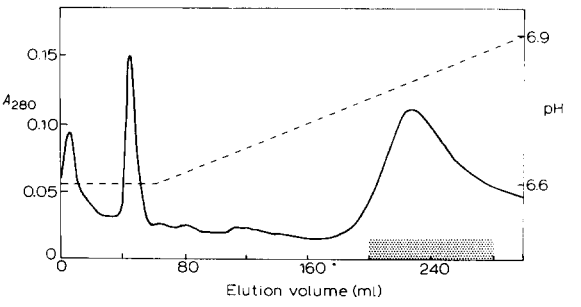

Fig. 3. Column chromatogram with hydroxyapatite of the DEAE-Sepharose fraction. The column size was 1.6 cm diameter and 20 cm height. The effluent contained 0.125 M sodium phosphate buffer, pH 6.6, at elution volumes from 0 to 60 ml, and the same buffer but with a linear pH gradient from 6.6 to 6.9 at elution volumes from 60 to 300 ml. Fractions at elution volumes from 200 to 280 ml indicated by dots were collected as the purified 33-kDa protein fraction.

thus prepared was completely free of contaminants (Fig. 1e), and amounted to 6 mg. The yield of the 33-kDa protein, on the basis of the starting material (thylakoid membranes), was 16%.

Further characterization of the 33-kDa protein

The 33-kDa protein prepared from the acetone powder of thylakoid membranes was very similar to that prepared from PS II particles in the previous study [1] in the physicochemical and chemical characteristics such as molecular mass, isoelectric point, absorption spectrum and the effect of 2-mercaptoethanol on the discontinuous and the SDS-polyacrylamide gel electrophoresis. The amino acid composition of the 33-kDa protein is presented in Table I. The contents of the minor amino acids were determined more precisely than in the previous study [1]. The nearest integers of half-cystine, methionine and tryptophan, were 2, 1 and 1, respectively. The occurrence of two half-cystine molecules explains the effect of 2-mercaptoethanol on the mobility in the discontinuous and SDS-polyacrylamide gel electrophoresis [1]. The value for the polarity index of the protein calculated from the amino acid composition according to Capaldi and Vanderkooi [16] was 49%. This value suggests that the protein is very hydrophilic as a membrane protein.

On the basis of the dry weight and the intensity of light absorption, $A^{1\%}_{1cm}$ at the absorption maximum, 276 nm, was determined to be 6.8. The extinction coefficient at 276 nm was calculated to be 22000 $M^{-1} \cdot cm^{-1}$ by using the values for $A^{1\%}_{1cm}$ and the molecular mass of 33 kDa.

Peptide mapping of the 33-kDa protein

In the SDS-polyacrylamide gel electrophoresis of thylakoid membranes from spinach chloroplasts, the band at 32 kDa was most densely stained in the molecular mass region from 30 to 35 kDa (Fig. 1a and b). In order to examine the identity of the 32-kDa polypeptide and the 33-kDa protein, we compared them by peptide mapping. Limited proteolysis was performed with the protease from *S. aureus*, and the digested samples were subjected to SDS-polyacrylamide gel electrophoresis. After proteolytic digestion of the 32-kDa polypeptide and the 33-kDa protein for 10 min, seven major fragments at 29, 26, 23.8, 23.2, 22, 21.5 and 9 kDa, and a few minor fragments around 15 kDa were discernible (Fig. 4b and g). With increasing digestion period, the band at 32 kDa as well as the bands at 29, 26, 23.8, 23.2 and 22 kDa became faint (Fig. 4c–e and h–j). On the other hand, the fragments at 21.5 and 15 kDa became intense and new fragments emerged at 22.7, 18, 13.5, 12.5 and 10 kDa and below the 9-kDa fragment. The modes of changes in the mapping pro-

TABLE I

AMINO ACID COMPOSITION OF THE 33-kDa PROTEIN OF SPINACH CHLOROPLASTS

The 33-kDa protein was hydrolyzed in 6 M HCl at 110°C for designated periods. Analysis was carried out with an amino acid analyser (Hitachi KLA-5). Values are expressed as mol amino acids/mol 33-kDa protein.

Amino acid	Period of hydrolysis			Nearest [a] integer
	24 h	48 h	72 h	
Lysine	28.3	29.2	29.6	29
Histidine	0.0	0.0	0.0	0
Arginine	7.5	7.3	7.8	8
Aspartic acid	26.3	26.1	25.9	26
Threonine	23.7	23.3	22.9	24 [b]
Serine	22.9	21.9	21.1	24 [b]
Glutamic acid	39.5	39.4	39.3	39
Proline	18.3	18.4	18.1	18
Glycine	36.4	36.0	36.0	36
Alanine	17.7	17.5	17.8	18
Half-cystine				2 [c]
Valine	21.7	22.8	23.3	23 [d]
Methionine	1.0	1.0	1.0	1
Isoleucine	9.1	9.5	9.7	10 [d]
Leucine	20.6	20.8	20.6	21
Tyrosine	9.7	9.6	9.7	10
Phenylalanine	15.8	15.7	15.8	16
Tryptophan				1 [e]
Total amino acids				306
Polarity index [f]				49%

[a] Average of three values of different hydrolysis periods unless designated by another superscript.
[b] Value obtained by extrapolation to zero hydrolysis time.
[c] Value for 24-h hydrolysis of the protein after oxidation with performic acid [14].
[d] Value for 72-h hydrolysis.
[e] Determined by a spectrophotometric method [15].
[f] Calculated according to the method of Capaldi and Vanderkooi [16].

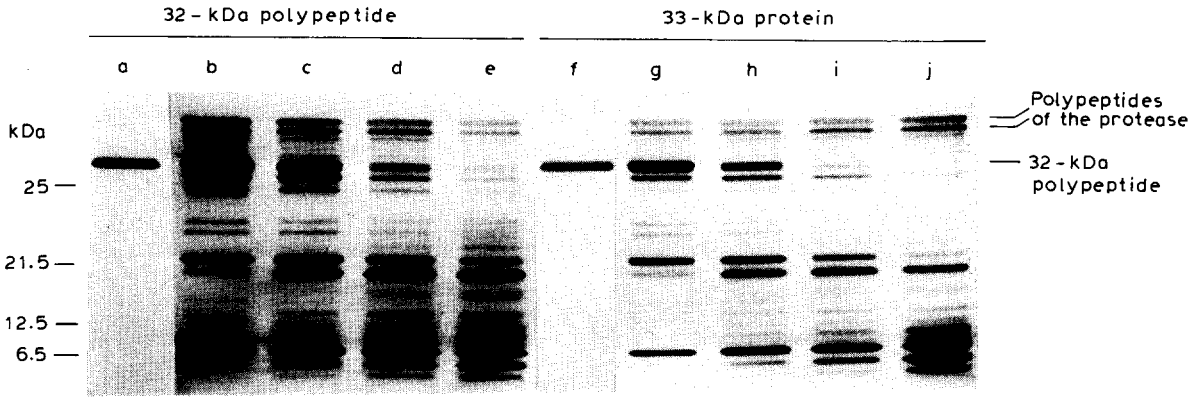

Fig. 4. One-dimensional peptide mapping of the 33-kDa protein and 32-kDa polypeptide. The 33-kDa protein was subjected to the first SDS-polyacrylamide gel electrophoresis. A zone of polyacrylamide gel containing the 33-kDa protein band was excised, and the protein was extracted from the excised gel. The acetone powder of thylakoid membranes was also subjected to the first SDS-polyacrylamide gel electrophoresis. A zone of gel containing the 32-kDa polypeptide band was excised and the polypeptide was extracted. After the 33-kDa protein and 32-kDa polypeptide thus extracted were digested by the protease from *S. aureus* for designated periods, they were subjected to the second gel electrophoresis. (a–e) Changes in peptide mapping with the protease of the 32-kDa polypeptide in the acetone powder. (f–j) Changes in peptide mapping with the protease of the 33-kDa protein. (a, f) No addition of protease. (b, g) 10 min proteolysis. (c, h) 30 min proteolysis. (d, i) 60 min proteolysis. (e, j) 120 min proteolysis. The molecular mass range is indicated in kDa. Proteins used as molecular mass standards were chymotrypsinogen A (25 000), trypsin inhibitor (21 500), cytochrome *c* (12 500) and aprotinin (6500).

files were very similar between the 32-kDa polypeptide and the 33-kDa protein.

When the 32-kDa polypeptide and the 33-kDa protein were treated with various concentrations of the protease, very similar changes in the profiles of peptide mapping were observed (data not shown). These findings suggest that the 32-kDa band in the SDS-polyacrylamide gel electrophoresis of the thylakoid membranes is entirely due to the 33-kDa protein.

The content of 33-kDa protein in the chloroplasts

Since the 32-kDa polypeptide in the thylakoid membranes was due to the 33-kDa protein, estimation was possible for the content of 33-kDa protein in the thylakoid membranes by comparing the staining intensity of the 32-kDa band with that of purified 33-kDa protein on the polyacrylamide gel. In the chloroplasts, the molar ratio of chlorophyll to the 33-kDa protein was 300 ± 60. This value indicates that one photosynthetic unit, which contains about 400 chlorophyll molecules [17], possesses one or two molecules of the 33-kDa protein.

Discussion

The study presented here indicates that the 33-kDa protein can be extracted from the acetone powder of the thylakoid membranes of spinach chloroplasts and purified by column chromatography on DEAE-Sepharose CL-6B and hydroxyapatite. The yield of the 33-kDa protein from that in the thylakoid membranes was 16%. In the previous study [1], we purified the 33-kDa protein by isoelectric focusing from the extract of PS II particles prepared from the thylakoid membranes. In this case, the yield of the protein from that in the thylakoid membranes is calculated from the result in Ref. 1 to be only 1.4%, on the basis that the molar ratio of chlorophyll to 33-kDa protein is 300 : 1 in the thylakoid membranes. This small value for the yield may be interpreted in part by the very low recovery of membanes in PS II particles. Thus, the method described in this study produces an about 10-fold larger amount of the 33-kDa protein than in the previous one.

Judged from its amino acid composition, the 33-kDa protein is very hydrophilic. We have al-

ready elucidated that the 33-kDa protein is firmly bound to the membrane [1]. The apparent incompatibility between the membrane-binding nature and the hydrophilicity of the protein can be interpreted by one of the following possibilities: Firstly, the 33-kDa protein is anchored by a hydrophobic membrane protein in the membrane-bound form. Secondly, the 33-kDa protein, though not tightly bound to the membrane, is confined in the intrathylakoid space. Thirdly, the 33-kDa protein exposes the hydrophobic amino acids on the surface of the protein in the membrane-bound form.

Silverthorne and Ellis [18] studied the 32-kDa polypeptide in the peptide mapping using the same protease as that used in our present work. They visualized the fragments of 32-kDa polypeptide by an autofluorogram of ^{35}S, so that the fragments lacking both cysteine and methionine would not be detected. Since the 33-kDa protein contains only one molecule of methionine and two molecules of cysteine (Table I), they must have detected only a part of the peptide fragments.

The function of the 33-kDa protein is not well established. The 33-kDa protein may be the same as the 32-kDa polypeptide studied by Siddell and Ellis [2] which is synthesized in the chloroplasts of greening pea leaves. This 32-kDa polypeptide is synthesized in isolated intact chloroplasts of maize, pea and spinach [3,5,6]. Grebanier et al. [3,4] have shown that a polypeptide having a molecular mass of 34.5 kDa is synthesized in the chloroplasts of greening maize and is processed to the 32-kDa polypeptide.

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