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Purification and Partly Characterization Apo-CP43 from *Escherichia Coli*

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ABSTRACT

CP43 is a chlorophyll binding protein, which is also the internal antenna system of photosystem II located in the thylakoid membrane of plants. To study the functional and structural aspects of apo-CP43, apo-CP43 was expression in E. coli BL21 (DE3) in the soluble state, and the optimal induced time for IPTG was 4 h. After apo-CP43 was purified by Ni-chelating column, gel filtration and Mono Q column, and the purified protein can be used in spectroscopy study. The intrinsic fluorescence experiments indicated that a maximum emission about 340.0 nm for apo-CP43 when λ_{ex}=295 nm, suggesting a relative polarity in the microenvironments of the tryptophan residues in the apo-CP43. While a maximum emission about 341.0 nm for apo-CP43 was obtained when λ_{ex}= 278 nm. The CD spectra of the purified apo-CP43 showed negative bands at 205 and 222 nm. The secondary structures analysis suggested that apo-CP43 has 39.11% α-helix with 28.90% random coil.

Keywords: Apo-CP43, Expression, Purification, Emission spectrum, CD

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INTRODUCTION

CP43 is an inner antenna protein of photosystem II (PSII), providing a conduit for excitation energy transfer from the exterior antenna of the PSII to the reaction center [1]. CP43 involves in light induced transmembrane charge separation, and also acts as an inner light-harvesting antenna system [1, 2]. CP43 is encoded by the *Psb* C gene (in chloroplast) and its apparent molecular mass, judged by SDS-PAGE, is 43 kDa, the calculated molecular mass for spinach is 50 kDa [3, 4].

As well known, CP43 possesses six transmembrane *α* –helices (I, II, III, IV, V, VI) and these helices were separated by five extrinsic loop domains (designated extrinsic loops A–E) , both of the N- and C-termini are stromally exposed [5-7]. The loop E has many function sites and it is research hot spot, the site-directed mutagenesis of basic arginine residues 305 and 342 and deletion mutations in the loop E of CP43 would affect oxygen-evolving activity [8]. A deletion mutant of *Psb* C in the PSII, which could lead to only part of PSII could assembly and the oxygen-evolving activity is also affected [9].

As the inner antenna protein for the PSII, CP43 interacts with proteins associated with the sites of water oxidation. Biochemical and genetic evidence indicates that CP43 may help form the binding site(s) for several extrinsic proteins to PSII. This assertion appears to be supported by the recent 3.8 Å crystal structure of the *Synechococcus elongatus* PSII [10]. CP43 lies on either side of the heterodimeric D1/D2 reaction center complex and is adjacent to D1 [11].

CP43 only binds chlorophyll *a* (13) and *β*-carotene (3 or 4) [12, 13] and the chlorophylls are located in symmetry-related positions. Additionally, in inner antenna the chlorophylls are roughly distributed in two layers near the stromal and lumenal sides of the thylakoid membrane, both of them are closely associated with the photochemical reaction center [14]. *β*-carotene locates at the inter-faces between subunits [12, 15] , like CP43 and Psb K / Psb Z [16].

CP43 is a membrane protein, in the previous studies, the membrane proteins of LHCs for PSⅡ were expressed by inclusion bodies [17-19]. Here we describe a novel approach of soluble expression and isolation for apo-CP43 in *Escherichia coli* BL21. However, it remains unclear whether the apo-CP43, over expressed in *Escherichia coli*, has the similar spectroscopic characteristics compared with the native CP43. To address this issue, this research isolated apo-CP43 with different purification step from *Escherichia coli*, and got the data about fluorescence and CD.

MATERIALS AND METHODS

Materials

Escherichia coli (*E. coli*) BL21 (DE3) and the vector pET-18a were obtained from

Novagen (Madison, WI). Yeast extract and trypton were purchased from Oxide. The Ni-chelating column was obtained from Germany. The Amicon Ultra-15 centrifuge tube with a filter of 5000 Da was purchased from Millipore. Tris-base, glycine, IPTG, SDS, and mercuric chloride were products of Sigma. Other chemical reagents were of analytical grade, and Milli-Q water was used to prepare the solutions.

Methods

Soluble expression of apo-CP43 in BL21

Apo-CP43 was overexpressed in the BL21 strain of *E. coli*. The cells were grown on 50 ml sterilized medium LB which had been supplemented 30 mg/ml kanamycin (Sigma). After overnight (12-16 h) growth of 37 ºC this culture was transferred to a 1 liter flask containing 500 ml sterilized medium. Cells were grown with moderate shaking (200 rpm) to a density of OD_{600}= 0.6 before protein expression was induced with 1 mM IPTG (Isopropyl-thio-*β*-D-galactopyranoside), and the cells were in exponential phase, the temperature for protein induction was 27 ºC. Cells were harvested by centrifugation at 4 ºC for 10 min at 6,000*g* after cells reaching stationary phase. The cells precipitate for 500 ml culture were resuspended in 25 ml cold buffer A, containing (20 mM Tris-HCl, 25 mM imidazole, and 500 mM sodium chloride, pH 8.0), and passed through two times of sonication to release protein in the cells. This suspension was centrifuged for 20 min at 10,000 *g* for two times and the supernatant contained apo-CP43.

Affinity chromatography

The modified method of immobilized metal-ion affinity chromatography (IMAC) [20] of Ni-chelating column connected to FPLC system to purify apo-CP43. The column was connected to ÄKTA FPLC system with an A280 detector connected to a recorder, and equilibrated with at least 10 column volumes of buffer A. Buffer B, containing (20 mM Tris-HCl, 500 mM imidazole, and 500 mM NaCl, pH 8.0) was used during imidazole- gradient elution for apo- CP43. The protein solution was loaded onto the column at a flow speed of 0.5 ml/min and the first time of penetration would be loaded to the column again with the same flow speed. The weakly bound proteins were washed from the resin with buffer A. The recombinant histidine-tagged protein was eluted by increasing the imidazole concentration from 100 mM imidazole to 300 mM imidazole in 1 column volume. The high imidazole concentration could avoid the nonspecific protein to bind the column. The eluted fractions were analyzed by SDS–PAGE and fractions.

Gel filtration

The further purified procedure used the gel filtration to remove the imidazole and the nonspecific protein, HiPrep 16/60 Sephacryl S-300 column can connect to the FPLC system. The fractionation range of this column for globular proteins is between 1000-5000 Da. The flow speed was 1.0 ml/min. This column should be equilibrated by 10 volumes

distilled water, and then washed by 10 volumes of Tris-HCl buffer (pH 8.0). The eluted solution contained salt and some nonspecific protein.

Anion exchange chromatography

After purification by gel filtration, there was still some non-protein in the protein solution, then used anion exchange chromatography to get the purified apo-CP43. Mono Q HR 5⁄5 was a pre-packed column, which was designed for fast, high solution anion exchange separations of proteins. Separations of substances with molecular weights up to 10^7 have been carried out successfully and it can connect to the FPLC system. Finally, after different purification steps, the apo-CP43 concentration in protein solution was too low for the subsequent experiment, and the solution of apo-CP43 can be centrifuged at 5000 rmp in the Amicon Millipore centrifuge tube. The ultrafiltration could concentrate the necessary protein content. All experiments were performed in a cold room (4 ºC).

SDS-PAGE

The protein from every purified step should be analyzed by modified SDS-PAGE which was described by Laemmli [21]. The concentration of stacking and resolving gel was 5%, 12.5%, respectively. Protein bands were stained by Coomassie brilliant blue. The markers were from Sigma.

Protein determination

Protein concentration was determined using the method of Markwell [22], which is a modification of the method of Lowry, bovine serum albumin was used as a standard.

Spectroscopy at room temperature

For room temperature, the purified protein solution was excited at 278 and 295 nm, and the record in the region of 310-400 nm. The emission spectra were recorded by a Hitachi F4500 fluorescence spectrophotometer, in 1 cm optical path quartz cuvettes, the excitation slit width was set 5 nm. Every spectrum was the average of three spectra and the spectrum of the blank buffer was subtracted. Circular dichrosim (CD) spectra were obtained at room temperature with an AVIV Model 400 dichrograph with Tris-HCl buffer (pH 8.0), the far-UV CD spectrum for apo-CP43-WT in quartz cuvettes with 2 mm path length was recorded in the region of 200-260 nm. Spectrum was recorded at a 0.2 nm/s scanning speed. Samples were in 2 mm optical path quartz cuvettes. Every spectrum was the average of three spectra and the spectrum of the blank buffer was subtracted.

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RESULTS AND DISCUSSION

Expression in E.coli and purification of His-apo-CP43

The cells were grown on sterilized medium LB which had been supplemented kanamycin. Cells were grown with moderate shaking (200 rpm) to a density of OD_{600}= 0.6, 1 mM IPTG was added into the medium to induce the expression of apo-CP43. After induction of 2, 3, 4 and 5 h, and then extracted protein solution samples. The analysis of SDS-PAGE indicated that the amount of expressed apo-CP43-WT would increase with prolonged induction time. The protein band above the mass molecular 45 kDa was gradually enhanced, which was monomer apo-CP43-WT. The dimmer of apo-CP43 protein band was above the BSA, the amount of monomer and dimmer apo-CP43 reached the highest when the induced time was 4 h. The mass molecular of apo-CP43-WT protein band with consistent with the size of the putative fusion protein.

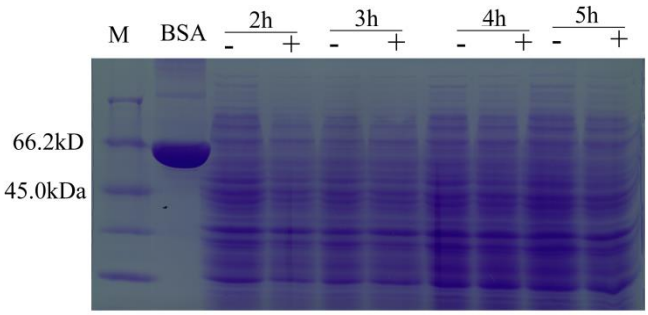

Fig. 1: Apo-CP43-WT was induced by IPTG for 2, 3, 4, 5 h, respectively

M. Marker, BSA. Bovine serum albumin (66 kDa), -. Uninduced BL21 supernatant, +. Induced BL21 supernatant.

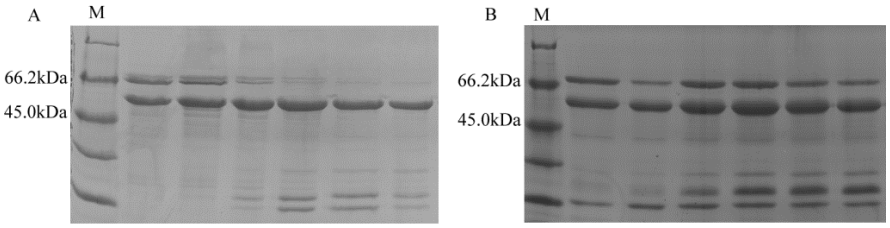

Fig. 2. SDS-PAGE analysis of apo-CP43-WT and the mutant H237F after purified by the Ni-chelating column

A. apo-CP43-WT, B. apo-CP43-H237F, M. Marker, the sample in the lane from 2 to 7 was the protein from the gradient-elution with time.

After the apo-CP43 protein was induced in the cells for 4 h, the SDS-PAGE analysis of apo-CP43-WT and the mutant H237F after purified by the Ni-chelating column were showed in Fig. 2. For apo-CP43-WT, the protein band was between the mass molecular 45. 0 kDa and 66.2 kDa, which was about 50 kDa and had been identified by western, blot (data not shown). The amount of dimmer for apo-CP43-WT was little, and there were a few contaminating protein bands presenting and the amount was also little, which needed another purification step to remove the contaminated. For the mutant H237F, the amount

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of induced protein was similar with the WT, but the quantity of dimmer was more than that of WT, the amount of non-specific protein had also increased, especially for the protein band above the marker for mass molecular 25 kDa. This because that the affinity for the mutant H237F to the Ni-chelating column was weaker than that of WT.

The protein solution from the Fig. 2A had a few contaminating protein bands and imidazole, which had great influence to the spectroscopy and CD for apo-CP43. Gel filtration can eliminate the salt of imidazole and remove some of the non-specific protein, but the content of apo-CP43 would also be decreased and the dimmer of apo-CP43 still existed. Though the amount was pretty small, the dimmer also can affect the spectroscopy and CD, so the Mono Q column was used to eliminate the dimmer and other protein bands. From the Fig. 3B, the contaminating proteins bands and dimmer were eliminated. However, the protein content for WT was too low to the following experiment. The protein solution should be concentrated by ultrafiltration of Amicon Millipore, most of the biomolecules whose mass molecular was less than 5000 Da would be removed. All the content for protein solution from each purification step would be analyzed by modified Lowery method.

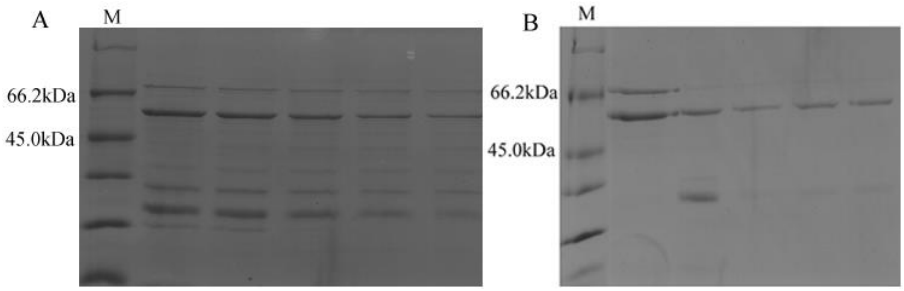

Fig. 3. SDS-PAGE analysis of apo-CP43-WT after purified by the gel filtration and Mnono Q column

A. for gel filtration, B. for Mnono Q column, M. Marker

After each purification step, the protein content was decreased gradually and the contaminating protein was also eliminated in the step of Mono Q column. The subsequent experiments were about the structure for apo-CP43 of spectroscopy and CD, the protein content was low, and then concentrated by ultrafiltration. The protein content of 0.40 mg/ml can be used in the following research.

Table 1: The content of apo-CP43-WT after each purification step.

Purification step	Total volume (ml)	Protein content (mg/ml)
Crude extract	80	3.57
Ni-chelating column	15	0.63
Gel filtered	8	0.25
Mono Q column	5	0.15

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The intrinsic fluorescence emission spectroscopy of apo-CP43-WT

The intrinsic fluorescence emission spectroscopy can reveal the structural changes in the microenvironment around the chromophores in a protein [23, 24]. The main chromophores in apo-CP43-WT are the seventeen tryptophan residues and the fifteen tyrosine residues [5-7]. The representative fluorescence emission spectra of apo-CP43-WT with λ_{ex} = 278 nm and λ_{ex}= 295 nm were shown in fig. 4A and B, respectively. The fluorescence emission spectrum with λ_{ex}= 278 nm mainly indicates the structural changes around both of the tryptophan and the tyrosine residues in protein, while the fluorescence emission spectrum with λ_{ex} = 295 nm only indicates the structural changes around the tryptophan residues in protein [23, 24]. The maximum emission wavelength of the fluorescence emission spectrum for apo-CP43-WT with λ_{ex}= 278 nm (Fig. 4A) and λ_{ex}=295 nm (Fig. 4B) were located at about 341.0 and 340.0 nm, respectively. The fluorescence intensity for the maximum emission wavelength was 483.6 and 208.6 a.u.

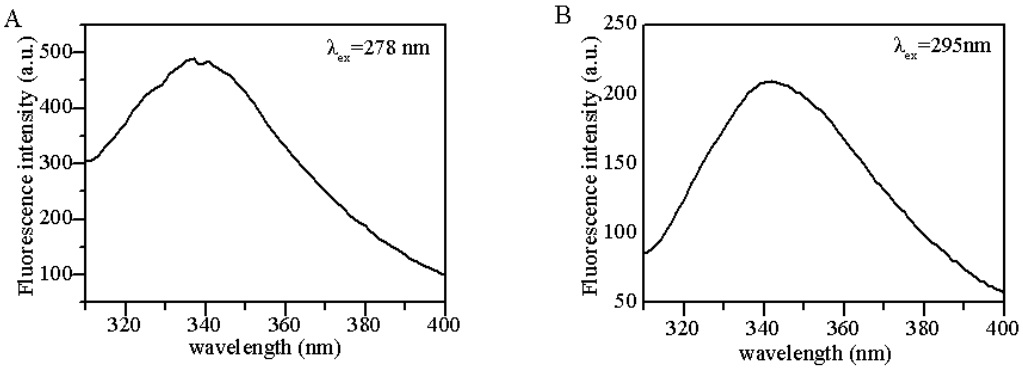

Figure 4: Fluorescence emission spectra of apo-CP43-WT at room temperature for extrication wavelength of 278 and 295 nm.

The far–UV CD of apo-CP43-WT

The representative CD spectrum for apo-CP43-WT was shown in Fig. 5. There were two distinct negative signals in the 208 and 222 nm, which were pretty similar to the far –UV CD of native CP43 with the pigment [25]. The lowest negative signal was at 208 nm. For the secondary structures of apo-CP43-WT expressed in *E.coli*., and the apo-CP43 was separated by coiled-coil helix dimmers (I, II), (III, IV) and (V, VI) [26]. Apo-CP43-WT has 473 amino acid residues, which has 10 histidine residues in protein [3], metal ions can bind to the exposed imidazoles of histidine residues [24], so during the CD experiment, the care must be taken to the metal ions and prevented them to contaminate protein sample. The secondary structures of apo-CP43-WT, α-helix possessed the ratio with 39.11% and the second was random coil.

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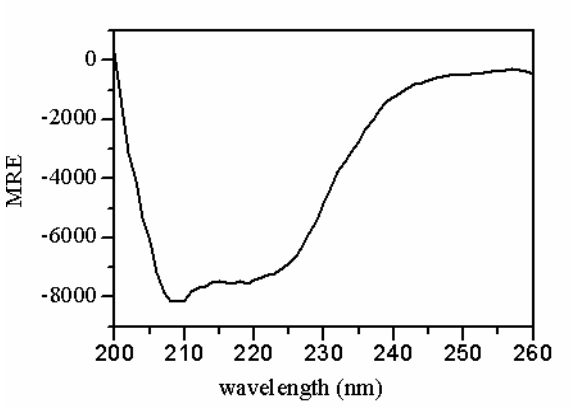

Fig. 5 The representative CD spectrum for apo-CP43-WT at 20 ºC.

Table 2 The second structures of apo-CP43-WT revealed by the CD spectrum.

Secondary structures	
α-Helix	39.11%
β-Sheet	15.27%
β-Turn	16.72%
Random coil	28.90%

CONCLUSION

Considering the important biological activity and complicated structure of the core antenna protein of apo-CP43, we described a novel approach to overexpress and purify the protein with BL21 (DE3) in this study. In the previous researches, they used inclusion bodies to over express membrane proteins of LHCs, like CP24, CP26 and CP29 [17-19]. The study found that the optimal induced time for apo-CP43 was 4 h, and the contaminating proteins and the dimmer of apo-CP43 were difficult to be eliminated, because they had the similar affinity to the purified column. The maximum emission wavelength of the fluorescence emission spectrum for apo-CP43-WT with λ_{ex}=278 nm and λ_{ex}=295 nm were located at identical locaton. The far-UV CD of apo-CP43-WT showed the most of secondary structure for it was α-helix.

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