Purification, crystallization and preliminary crystallographic investigations of selenium-containing phycocyanin from selenium-rich algae (*Spirulina platensis*)

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Abstract The selenium-containing phycocyanin from the selenium-rich algae (*Spirulina platensis*) has been crystallized in two crystal forms by the hanging-drop vapor diffusion techniques. A chromatographic procedure of gel filtration and anion exchange was used for purification. Form I crystal with space group $P2_1$ and cell parameters a =108.0 Å, b = 117.0 Å, c = 184.0 Å, β = 90.2° and 12($\alpha\beta$) units in the asymmetric unit was obtained by using $(NH_4)_2SO_4$ as precipitant. These crystals diffract up to 2.8 Å. Form II crystal obtained by using PEG4000 as precipitant belongs to space group $P6_3$ with unit cell constants a = 155.0 Å, c = 40.3 Å, γ =120.0° and one($\alpha\beta$) unit in the asymmetric unit. The crystals diffract beyond 2.9 Å. The possible stacking forms of phycocyanin molecules in the first crystal form were discussed.

Keywords: phycocyanin, crystallization, X-ray diffraction, the study of crystallography.

Selenium is an essential trace element of humans and animals[1]. In 1973, Rotruck et al. discovered the first selenoenzyme, glutathione peroxidase[2]. The discovery has led to increasing awareness of nutritional importance of selenium and provided a marked stimulus to investigation of the nutritional and biochemical roles, and antitumor mechanism of the element. At present, more and more selenoenzymes and selenoproteins have been isolated from animals and microorganisms and their functions have been identified. Furthermore, one of the striking new developments in the field of selenium biochemistry has stemmed from the finding that selenocysteine is encoded by codon UGA in mRNA during selenoprotein synthesis[3, 4].

On the other hand, because of the medical functions of organic selenium, selenium accumulation in plants has been investigated to transfer inorganic selenium to organic selenium that can be easily absorbed by human body. Many investigators found that the level of selenium in plant increased after selenium fertilizers supplied by roots or foliar spraying and selenium was involved in plant metabolism such as antioxidization and protein synthesis. It has been reported that selenium follows the metabolic route analogous to S in plant and the selenium-containing

compounds are synthesized from R-Se-R′ [5,6]. Sun et al. demonstrated that the peroxidase of shoots in winter wheat (*Triticum aestivum* L.) cultured in selenium medium contained selenium[7]. In addition, selenium-containing proteins in selenium-rich algae and selenium-rich yeast were also reported[8—10]. However, in spite of these indications above, there is no direct evidence at present concerning selenoprotein synthesis in plant.

Phycocyanin in red algae and blue-green algae is a macromolecular light-harvesting complex, which absorbs and transfers light energy to the photosynthetic reaction centers in the thylakoid membrane. Therefore, determination of the three-dimensional structure of phycocyanin will be helpful to providing a better understanding of photosynthesis mechanism. On the other hand, the introduction of phycocyanin as fluorescent tags of cell and molecules was followed by widespread application of these macromolecules in cell sorting, cell analyses, flow cytometry, fluorescent microscopy and immunoassay[11]. Up to the present, the crystal structures of some phycobiliproteins have been solved at high resolution such as B-PE from *Porphyridium sordidum*[13], R-PE from *Polysiphonia urceolata*[14,15], b-PE[16] and B-PE[17] from *Porphyridium cruentum*; PEC from *Mastigocladus laminosus*[18]; C-PC from *Mastigocladus laminosus*[19], C-PC from *Agmenellum quadruplicatum*[19], C-PC from *Fremyella diplosiphon*[20], C-PC from *Anabaena variabilis*[17]; APC from *Porphyra yezoensis*[21] and APC from *Spirulina platensis*[12] . Between 1995 and 1997, Moreno and Brejc et al. reported their investigations of phycobiliproteins from algae *Spirulina platensis*[11,12], but the three-dimensional structure of phycocyanin of this algae is still unknown.

Spirulina platensis is a nutritional and medical microalga, but there is little of selenium to be determined in the algae. However, the algae cultured in selenium medium are able to absorb inorganic selenium and synthesize selenium-organic compounds. The investigations have shown the selenium content of phycocyanin in the selenium-rich algae, but the incorporation of selenium into protein or the form in which selenium occurs in the protein is still unknown.

In this article we report the isolation, crystallization and preliminary crystallographic studies of phycocyanin from selenium-rich algae.

1 Experiments and results

1.1 The culture of selenium-rich algae

Cells of *Spirulina platensis* (seeds provided by Shenzhen Blue Algae Company) were grown according to Zarrouk medium (pH8.5—9.5) at 28—35℃ with a photoperiod of 12 h irradiance and 12 h darkness. Cultures were continuously bubbled with air. Light was provided by fluorescent tubes. The light intensity 50 $\mu Em^{-2} s^{-1}$ was used. After 3 days, the Na_2SeO_3 concentration of the culturing medium is up to 600 mg/L by adding Na_2SeO_3 in the medium. After 12 days, cultured cells were harvested. Then the algae dialyzed against H_2O were stored at −20℃ until use.

1.2 Isolation and purification of phycocyanin

10 g algae (dry weight) were suspended in 200 mL of 0.2 mol/L phosphate buffer, pH 7.0 and frozen at −20℃ several times until cells of frozen algae were all disrupted. The frozen cells were

centrifuged at 3000 r/min for 20 min (4℃) to discard aggregated material. Solid $(NH_4)_2SO_4$ was added to 20% of saturation in the resulting supernatant containing solubilized proteins, allowed to stand for 2 h and then centrifuged at 3000 r/min for 20 min (4℃) to discard the pellets. In the supernatants, solid $(NH_4)_2SO_4$ was added to 45% of saturation and then centrifuged at 3000 r/min for 20 min (4℃) to discard the supernatants. The pellets were resuspended in 0.2 mol/L phosphate buffer, pH7.0 and dialyzed overnight against the same buffer (4℃). The dialyzed phycocyanin-containing solution were applied to a column (60 cm×2.5 cm) of DEAE-52 pre-equilibrated with 0.05 mol/L phosphate buffer, pH7.0. After rinsing with a salt gradient solution, absorption spectra of the phycocyanin-rich solution were measured by using a UV-3000 spectrophotometer (fig. 1) and the protein solution was pooled according to A_{620} / A_{280}. The pooled phycocyanin-rich solution was applied to a column (60×2.5 cm) of Sephadex G-100. In the eluted phycocyanin-rich solution, a constant (A_{620} / A_{280}>3.5) was obtained. For the phycocyanin-rich solution (A_{620} / A_{280}>3.5), anion-exchange was performed using a FPLC (MonoQ) column. The elution containing phycocyanin was pooled and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE showed two bands in phycocyanin. By comparison with standards, the molecular weights were 19000 and 21500 u, corresponding to the α and β subunits of phycocyanin (fig. 2).

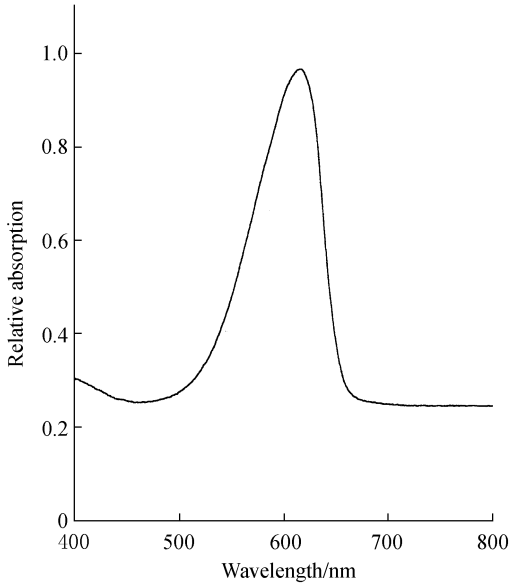

Fig. 1. Absorption spectrum of phycocyanin in *Spirulina platensis* (25℃).

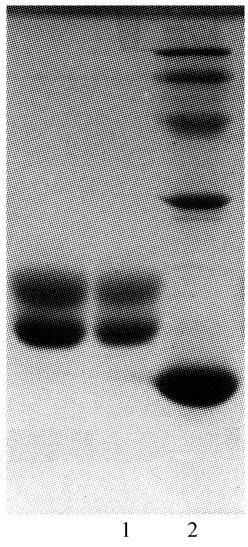

Fig. 2. SDS-PAGE of phycocyanin in *Spirulina platensis* from anion-exchange (MonQ) chromatography (FPLC). 1, C-PC-rich solution; 2, marker proteins (from top to bottom): phosphorylase B (94000), bovine serum albumin (67000), actin (43000), carbonic anhydrase (30000), α-lactalbumin (14000).

1.3 Se determination of phycocyanin

DAN-fluorescence spectral analysis[22]: To a flask containing the purified protein solution, add the blended HNO_3 and $HClO_4$ (HNO_3 : $HClO_4$ = 4 : 1) to oxidize organic substances and subsequently evaporate the solution over an oil bath. Once the organic substances are destroyed, evaporate the solution to approximately 5 mL of residues. After cooling to room temperature, pick up the residues with ddH_2O, add hydroxylamine hydrochloride, EDTA, and cresol red and adjust the solution to pH1—2. Then transfer the solution to the test tube. In the dark room, add 0.1% 2, 3-diaminonaphthalene to the tube and make the sample react for 5 min on a boiled water bath. When cool, transfer it to a separating funnel, add cyclohexane and shake it for 3 min, and leave it to stand for about 5 min in order to separate the phases. When the layers are completely separated, withdraw the organic phase with a measuring pipette and filter it into the measuring cuvette. The fluorescence intensity of the organic phase was measured by using an RF-5400 fluorescence spectrophotometer.

Equipment parameters were as follows: excitation wavelength 376 nm, emission wavelength 520 nm, aperture width 10 nm and cuvette, path length 1 cm.

To establish the calibration curve, use the selenium standard solution to prepare a series of concentrations in the range of 0.01—0.2 μg Se. Read off the corresponding selenium content from the calibration curve of selenium on the basis of the fluorescence intensity measured, taking the blank reading into account. Se content of phycocyanin is 86.8 μg Se/g protein.

1.4 Crystallization and X-ray diffraction studies

1.4.1 Form I. The crystals were obtained by hanging drop vapor diffusion method in dark room. The crystals can grow to 0.65×0.4×0.15 mm at room temperature after 4—5 weeks (fig. 3). The crystallization conditions are given in table 1. Diffraction data recorded on 170 images of 1° oscillation angle with 6 min exposure time were collected to 2.83 Å, using a scaled-tube generator (2 kW) and a Mar345 image plate system. The crystal to detector distance was 280 mm. All images were processed with DENZO[23] and SCALEPACK. The crystal belongs to group $P2_1$ with cell dimension a = 108.0 Å, b = 117.0 Å, c = 184.0 Å and β = 90.2°. The unit cell (2351737.5 $Å^3$) contains 24($\alpha\beta$) monomers, which corresponds to solvent content of 43%. There are 12($\alpha\beta$) monomers (M.W = 40.5 ku)

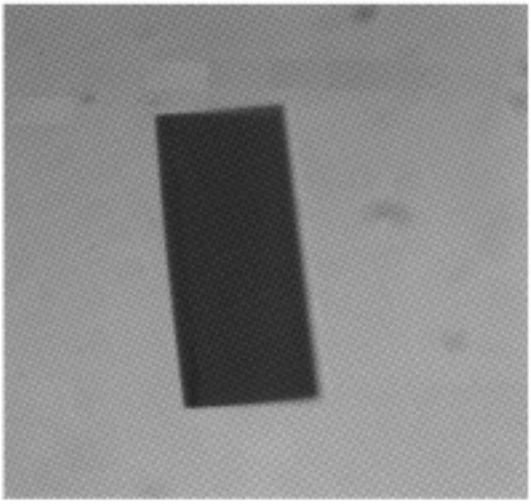

Fig. 3. The photography of form I crystal of phycocyanin from *Spirulina platensis*.

in the asymmetric unit. The data collection of form I crystal is given in table 2.

Table 1 The crystallization conditions of form I and form II crystal of phycocyanin from *Spirulina platensis*

	Form I	Form II
A solution	MPD 2.5% (*V*/*V*) saturated $(NH_4)_2SO_4$ 16%(*V*/*V*) saturated NaCI 6% (*V*/*V*) 0.1 mol/L Na_2HPO_4-NaH_2PO_4 pH 6.79	PEG4000 13% (*V*/*V*) $MgCI_2$ 0.4 mol/L Hepes 0.1 mol/L pH 7.37
B solution	protein 11.0 mg/mL 0.1 mol/L Na_2HPO_4-NaH_2PO_4 pH 6.79	protein 10.0 mg/mL Hepes 0.1 mol/L PH 7.37
Hanging drop solution	4 μL A solution +4 μL B solution	4 μL A solution +4 μL B solution
Diffusion solution	saturated $(NH_4)_2SO_4$ 25% (*V*/*V*) 0.05 mol/L Na_2HPO_4-NaH_2PO_4 pH 6.79	PEG4000 14% (*W*/*V*) $MgCI_2$ 0.4 mol/L Hepes 0.05 mol/L pH 7.37

MPD: 2-methyl-2,4-pentanediol.

Table 2 Data collection of form I and form II crystals

	Form I crystal	Form II crystal
Total observations	156183	72329
Unique reflections	59891	13448
Resolution/Å	3.5	2.93
Completeness (2σ cutoff)(%)	66.7	74
Average I / σ	7.3	8.2
Rmerge[a)]	0.07	0.08

a) Rmerge= $\Sigma_h \Sigma_i | I(h)_i - \langle I(h) \rangle | / \Sigma_h \Sigma_i I(h)_i$.

1.4.2 Form II. The crystal with the maximum dimensions 0.4 mm×0.4 mm×0.3 mm (fig. 4) has grown for 1—2 weeks at 17.5℃ in darkness. The crystallization condition is in table 1. Diffraction data on 220 images were collected on a Mar345 image plate system. The data collection parameters are: X-ray generator power 2 kW (50 kV, 20 mA), oscillation angle 1°, exposure time 8 min and crystal to detector distance 280 mm.The autoindexing indicated hexagonal system with cell dimensions a = 115.0 Å, c = 40.3 Å, and γ = 120.0°. Systematic absence $l \neq 3n$ was observed along 00l direction, and there were no systematic absences along h00 and 0k0 directions. Inspection of the Laue system (6/m) of the reciprocal space showed that the space group was $P6_3$. There is one ($\alpha\beta$) monomer in the asymmetric unit with a Vm of 3.45 $Å^3 \cdot u^{-1}$ and solvent content of 60%. The crystals can diffract to 2.83 Å, but the statistics given in table 2 show that the highest resolution available is 2.93 Å.

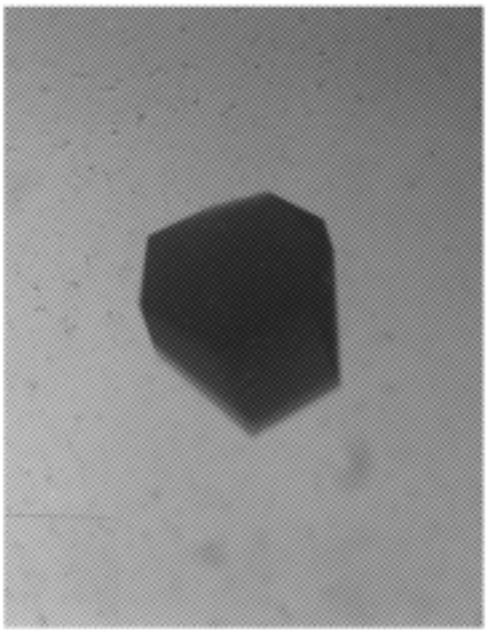

Fig. 4. The photography of form II crystal of phycocyanin from *Spirulina platensis*.

2 Discussion

The two mechanisms for ion absorption in plant are both active and passive uptake. According to an analogous way, inorganic selenium is taken up by algae. The recent experimental results suggested that in a high SeO_3^{2-} concentration range, SeO_3^{2-} is transported passively in algae and some of inorganic selenium could be converted to organic selenium compounds[24—26], and the other appeared yet to be in inorganic selenium molecule in cells of the algae. In this experiment, phycocyanin from selenium-rich algae dialyzed against ddH_2O were isolated and purified. The purified phycocyanin was dialyzed against ddH_2O for 3 days at 4℃ until no inorganic selenium in dialysate was measured. Accordingly, total selenium content in the phycocyanin (86.8 μg Se/g protein) was determined. However, the selenium bounded or chelated by phycocyanin, or the selenium as partial or complete replacement of S in phycocyanin, is unknown. We hope to answer the above questions and elucidate selenium nutrition mechanism of algae in molecule biological principle after solving the structure of phycocyanin.

The most commonly used technique is the determination of selenium by DAN-fluorescence spectral analysis. The method has a high sensitivity (range 6—1 ng) and is suitable for the microassay of selenium from biological material. In our experiment, the amount of Se from the leaves of cabbage (standard sample, GBWO8504, 0.083 μg Se/g) reach 0.080 μg Se/g and the returned coefficient of selenium from wheat powder is 98.6%. Therefore, these results have shown the accuracy of determination of selenium by the DAN-fluorescence spectral analysis.

Form I crystal was obtained with precipitant $(NH_3)_2SO_4$ in phosphate buffer. After optimi zations with pH, precipitant concentration and protein concentration, the mosaicity was highly indicated by diffraction image. After addition of 5% MPD, the crystal quality was improved and suitable for data collection. The β angle is 90.2° so that it is easy to determine mistakenly that the crystal system is orthorhombic. The intensities of spots related by mirror planes [m(*xy*0)], [m(*x*0*z*)] and [m(0*yz*)] were checked. It was found that the Rmerge for [m(*x*0*z*)] related reflection was 10%, while for the other two mirror planes the merging *R* factors were 31%. Along 0*k*0 direction there are systematic absences $k \neq 2n$. These results indicate that the space group is $P2_1$. According

to the reported crystal structures of phycobiliproteins, two $(\alpha\beta)_3$ trimers are aggregated face to face to form the $(\alpha\beta)_6$ hexamer. There is a triad axis in $(\alpha\beta)_3$ trimer which relates three $(\alpha\beta)$ monomers. In another allophycocyanin-linker complex, two $(\alpha\beta)_3$ trimers are arranged side by side, and the angle between the two trimers is approximately 90° [27]. For form I crystal, there are two $(\alpha\beta)_6$ hexamers or four $(\alpha\beta)_3$ trimers in the asymmetric unit. The elucidation of the monoclinic structure may be helpful to understanding how the $(\alpha\beta)_6$ hexamers or $(\alpha\beta)_3$ trimers are arranged. It is also possible to help us further know the light energy transfer route in phycobiliprotein. Now the crystallization condition is being optimized.

With regard to form II crystal, X-ray diffraction studies show that it belongs to the hexagonal system. The systematic absences of reflections indicate that the space group is $P6_3$, with one $(\alpha\beta)$ unit in the asymmetric unit and the unit-cell constants a = 115.0 Å , c = 40.3 Å and γ = 120.0° analogous to the crystal of phycocyanin from *Mastigocladus laminosus*.

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