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Crystallization of Phycobilinproteins of a Cyanobacterium *Calothrix elenkinii Koss*

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Abstract

Bilin-based fluorescent proteins, phycoerythrin, inducible phycocyanin, and constitutive phycocyanin, were isolated from cyanobacterium *Calothrix elenkiniii* by native gel electrophoresis. The purified proteins were crystallized in $MgSO_4$ precipitation solution. Isolation method of the bilin protein by native gel electrophoresis is easiest method, time consuming and pure, crystallization of these economically important phycobilin proteins is reliable.

Keywords: Cyanoacteria, phycobilinprotein, phycoerythrin, phycocyanin

Introduction

Red algae and blue-green algae (cyanobacteria) obtain their characteristic colors from a variety of pigments, including chlorophylls and carotenoids associated with transmembrane photosynthetic reaction centers, and abundant phycobilinproteins (PBPs) are the main components of their light-harvesting complexes [1,2]. Cyanobacteria naturally synthesize 4 different bilins, the most common of which are the blue colored phycocyanobilin (PCB) and the purplish-pink colored phycoerythrobilin (PEB). Studies of Complementary Chromatic Adaptation (CCA) have shown the existence of 2 distinct PBP types, the red-absorbing phycocyanin (PC) and the green-absorbing phycoerythrin (PE) content, when grown in red and green light conditions [3,4], achieved by changing the phycobilisome rod structure and polypeptide composition [5,6].

Algae are nutritious because of their high protein content and their high concentration of minerals, trace elements, and vitamins [7]. Additionally, PBPs are also widely used as natural colorants in food, cosmetics, and as drugs to ward off disease [8]. Especially, cyanobacterial phycocyanin (C-PC) has been patented for its usage in the treatment of autoimmune diseases, allergies, and cancers [9], and is widely used due to its economic benefits.

Earlier studies of cyanobacteria and other algae have been mainly focused on the x-ray crystallographic structures of the PBPs [10,11]. Crystallization is a chemical solid-liquid separation technique, in which mass transfer of a solute from a liquid solution to a pure solid crystalline phase occurs. It is more beneficial than other separation techniques, and can help in the formulation of drug chemicals into capsules; the dissolution of crystals can be well-characterized and, thus, allows for easier drug formulation. Additionally, as it provide possibly the most concentrated form of the chemical, the crystallized protein ensures its structural intactness, and offers the opportunity for further investigation of its structure or the composition of the extremely pure protein within the crystals. More than 80 % of the substances used in pharmaceuticals, fine chemicals, agrochemicals, food, and cosmetics are isolated in their solid form. Hence, study of the crystallization of these proteins is essential from a biochemical perspective.

Studies of the crystallization of the PBPs of cyanobacteria *calothrix ellenkinii Koss* are still lacking. The high phycobilin fluorescence present in this species can be used for fluorescent probes [12]. The method of separation of PBPs by chromatographic column is time consuming. Hence, we report here a method to purify bilinproteins using a 2 step native electrophoreses process, which is less time consuming and gives a more refined product. A preliminary study, with the objective of identifying a standardizing crystallization method for these proteins, was carried out.

Material and methods

Isolation of phycobilisomes

Algal cells of *C. elenkinii* were grown in BG_{11} medium [13] under red and green light conditions. The isolation of PBS from the algal cell was performed with high molar phosphate buffers [14]. The method was refined by the use of Lauryldimethylamine-oxide (LDAO) instead of Triton-X-100, and optimized for the PBS of complementary chromatic adapting (CCA) cyanobacteria by the addition of 10 % sucrose (wt/l) to the isolation buffer [15]. The gradients displayed were eluted separately, precipitated in 1.8 M potassium phosphate at pH 7.0, and stored at 4 °C [16]. The precipitated phycobilisomes were dissolved in distilled water and subsequently transferred to Tris/boric acid (50 mM/120 mM pH 7.9), 2 mM EDTA, and 20 % sucrose (wt/l), by gel filtration on PD-25 columns (Amersham Biosciences). The samples were stored at –20 ºC for further investigations.

Native PAGE

To isolate the subunit complexes in their native state from PBS of RL and GL, the electrophoresis method was consequently optimized [17]. Native-PAGE was performed under stabilized conditions with 7.19 % polyacrylamide (wt/v) slab gels with Tris/boric acid (50 mM/120 mM, pH 7.9) containing 2 mM EDTA and 10 % sucrose (wt/l). Gels were polymerized with 0.2 % tetramethylethylenediamine (l/l) and 0.03 % ammonium peroxodisulphate (wt/l). The separation of PBS was performed with a constant power of 20 W at 10 °C for 20 h. PBPs like PE, PC_i, PC_c, and AP bands were cut (can store at –20 °C) and subsequently used to isolate the linker free "trimeric" bilinprotein complexes under partially stabilized conditions with 6.5 % polyacrylamide (wt/l) (slab gels) with Tris/boric acid (50 mM/120 mM, pH 7.9) containing 2 mM EDTA and 7 % sucrose (wt/v) [18]. Electrophoresis was performed at a constant power of 15W (04300 $V \times h^{-1}$) at 15 °C for 15 h.

Phycobilinprotein bands, like PE, PC_i, and PC_c, were cut and eluted from the gel for at least 3 h under continuous stirring in a 10-fold volume of Tris/boric acid (50 mM/120 mM, pH 7.9) [16]. Elution was centrifuged for 60 min at 70,000 rpm, and the supernatants was filtered through a 0.22 µm poly(vinylidene)difluoride membrane [19], subsequently concentrated by ultrafiltration with Centricon YM-30 (Pall filtron) at 5,000 rpm at 10 °C, and stored at –20 °C for further studies.

Crystallization of the phycobilinprotein complexes PE, PC_c, PC_i, and APC

The PBPs were crystallized by the vapor diffusion hanging-drop method [20]. The samples were transferred into 100 mM Tris/Boric acid, pH 7.9, by gel filtration, and the concentration of each sample was adjusted to 10 mg/mL by ultra-filtration. Approximately 7.5 % poly ethylene glycol (PEG) 6,000 (wt/ml) and 100 mM $MgSO_4$ in 100 mM Tris/Boric acid, pH 7.9, served as a precipitation solution. Each crystallization drop contained proteins (15 mg/ml), and precipitating solutions were in the ratio of 1:1 and 2:1. The reservoir plates were filled with 300 µL of precipitation solution and kept at 17 ºC in darkness for crystal growth. After 2 weeks, the crystallized bilinproteins were taken for further analysis.

Result and discussion

Complementary chromatic adaptive behavior of *C. elenlinii* is used in our study, to enhance the quantity of a particular protein synthesis, such as phycoerythrin under green light and constitutive and inductive phycocyanins under red light conditions. The applied gel strength and separation method has been shown to be suitable for the isolation of highly purified trimeric "native" bilinprotein complexes between 50, 000 and 500, 000 Daltons [17]. Nevertheless, stabilization of these phycobilinprotein (PBP) complexes was obtained by a moderate pH of 7.9, with a high buffer concentration of 120 mM Tris-Boric acid and a constant temperature at 15 °C. The modified sample preparation and the electrophoresis conditions yielded a better resolution of the "trimers" within the gels of a nearly complete dissociation of the phycobilisomes (PBS) into PBP complexes without rod linkers.

During our crystallizations study, several factors favored the growth of crystals. These were: 7.5 % PEG 6000, Tris/Boric acid buffers, pH about 7.9, using the precipitant $MgSO_4$. One successful strategy found was to use essentially the same precipitants, buffers, and salts for all the proteins, which had dramatic effects on crystal habits or shapes (**Figure 1**), with overall dimensions between 0.1 and 0.4 mm of crystal growth within 1 - 2 weeks. The rod shaped crystal of $PC{\cdot}L_R^{34}$ (**Figure 1A**) grew out of the red light PBS without any further purification. The content of the linker in the phycobilisomes has been confirmed by SDS-PAGE (data not shown). However, the some uncertainty remains from the analysis as to whether it is the larger or the smaller inductive rod linker. Still, under the conditions of a good separation of the subunits in the native PAGE, it was difficult to separate the linkers. The crystal packing arrangement and the space group is not be influenced by linker proteins [21].

Isolated trimeric PE 562 crystals of *C. elenkinii* (**Figure 1D**) have been obtained as a bright pinkish bow-like structure. Becker *et al*. obtained rod shaped PE 545 crystals from the cryptomonad alga, *Rhodomonas lens*. The crystals of PC_c 618 (**Figure 1B**) revealed hexagonal space groups [23], and PC_i 618 (**Figure 1C**) is of a rectangular and pentagonal shape. The crystallization of both PBPs complexes completely used the protein in the solution; our results confirm their structural intactness and their purity. As long as the crystals are formed and have a suitable crystal habit (shape), they are possibly suitable for structural analysis [22], which will improve product appearance and be fit to be used in the drug synthesis.

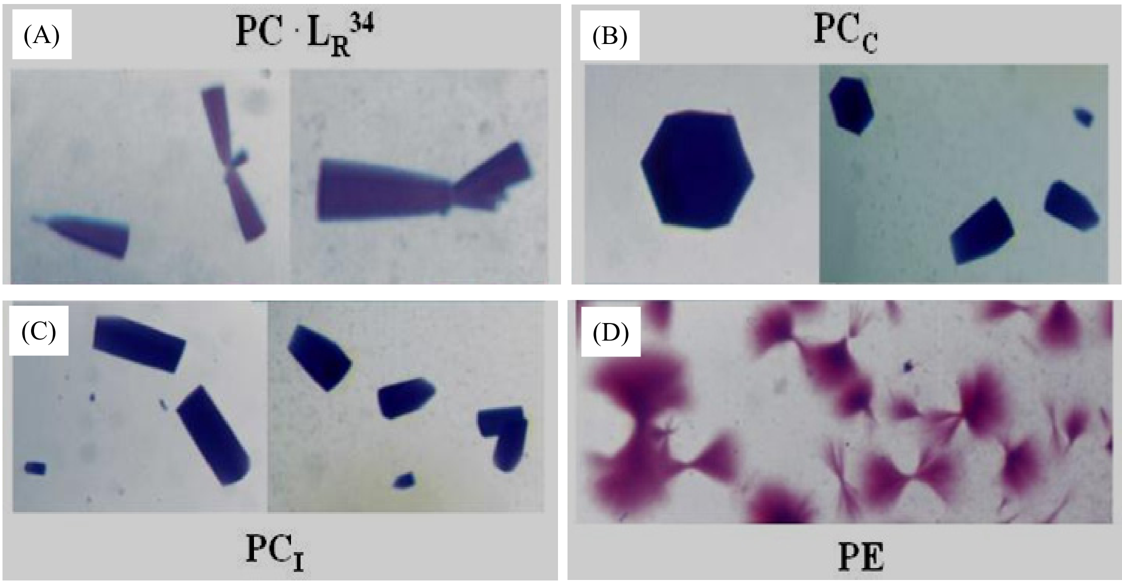

Figure 1 The phycobilinproteins isolated from *C. elenkinii*, grown by vapor diffusion in hanging drops containing 100 mM $MgSO_4$ in 100 mM Tris/Boric acid, pH 7.9 at 17 °C. The crystallization of the PBS complexes completely used the protein in the solution, confirm their structural intactness and their purity. (A) $PC.L_R^{34}$ - red light phycobilisome, phycocyanin with rod linker (rod shaped); (B) PC_c - constitutive phycocyanin (hexagonal shape); (C) PCi - inducible phycocyanin (rectangular and pentagonal shape); (D) PE - phycocrythrin (bow-like shape).

Conclusions

The literature indicates that the study of the phycobiliproteins of individual cyanobaceteria is novel [24]. Hence, with the results obtained above, we can conclude that the methodology for PBS- PE, PC_i, and PC_c separation in trimeric aggregation state and crystallization is useful for structural analysis. It is recommended that the production of the organism must be enhanced through culturing methods, as the proteins obtained from this cyanobacterium have immense commercial applications, especially in use as fluorescent tags.

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