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Combinational biosynthesis and characterization of a fluorescent 82β-phycocyanin of *Spirulina platensis*

GUAN XiangYu[1], ZHANG WeiJie[2], CHI XiaoYuan[2], LIN HanZhi[2,3], WANG JinFeng[2] & QIN Song[4*]

[1] *School of Ocean Sciences, China University of Geoscience, Beijing 100083, China;*
[2] *Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China;*
[3] *Graduate University of Chinese Academy of Sciences, Beijing 100049, China;*
[4] *Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai 264003, China*

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To biosynthesize fluorescent *Spirulina platensis* (*Sp*) β-phycocyanin (PC) in *Escherichia coli*, a BLASTP search for homologs of the *cpeS* gene, a chromophore lyase, was performed against the *Synechocystis* sp. PCC 6803 (*S6*) proteome. A highly homologous gene, *slr2049*, was obtained from the *S6* genome. Sites 82 and 153 in β-phycocyanin of *Sp* were modified by site-directed mutagenesis. Two recombinant expression vectors were constructed and transformed into *E. coli* BL21: (i) pCDF-*cpcB* (C153A)–*slr2049*–*sll0583*–*ho1*–*pcyA*; and (ii) pCDF-*cpcB* (C82I)–*slr2049*–*sll0583*–*ho1*–*pcyA*. Lyases encoded by the genes *slr2049* and *sll0583* catalyzed the linking of *Sp* 82β-PC to phycocyanobilin (PCB), and fluorescent CpcB (C153A)–PCB was generated. We present a strategy for the co-expression of multiple genes in a single expression vector to identify the function of an unknown gene. Recombinant phycobiliproteins produced on a large scale are promising fluorescent tags for diagnostics and pharmacology.

***Escherichia coli*, phycocyanin, fluorescence, recombinant**

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Cyanobacteria are primitive species with the capacity for oxygenic photosynthesis [1,2]. As a light-harvesting complex, phycobilisomes (PBSs) play a crucial role in energy absorption and transfer in the photosystems of most cyanobacteria [3–5]. There has been much research on PBSs because they have comprehensive applications in the fields of food, cosmetics, medicine, and biotechnology [6–9]. The natural phycobiliproteins (PBPs) in *Spirulina platensis* (*Sp*) have been under particular scrutiny because of their antioxidant properties [10–13]. PBPs can also be used as fluorescent tags and in light-dynamics therapy [4,14–16]. More stable PBS subunits are urgently needed for application in the above fields.

C-phycocyanin (C-PC) in *Sp* contains three phycocyanobilins (PCBs) located at α84, β84, and β155 [17]. The PCBs are covalently linked to cysteine residues through thioether bonding, which is mediated by corresponding lyases [4]. In *Escherichia coli*, the conversion of heme to biliverdin IX is catalyzed by the heme oxygenase H01 (encoded by the *ho1* gene) [18,19], then the conversion of biliverdin IX to PCB is catalyzed by the PCB ferredoxin oxidoreductase PcyA (encoded by the *pcyA* gene) [20,21]. Thus, biosynthesis of PCB can be achieved by co-expression of the *ho1* and *pcyA* genes in *E. coli* [22]. The α-subunit of C-PC contains a chromophore-binding site at position 82. Biosynthesis of holo-α-PC, which is the α-subunit of C-PC linked with PCB, is catalyzed by the lyases CpcE/CpcF [23]. The five genes required for the biosynthesis of *Synechocystis* sp. PCC 6803 (*S6*) holo-α-PC have been successfully co-expressed in *E. coli* using only a single expression vector [24]. In

*Corresponding author (email: sqin@yic.ac.cn)

contrast, the fluorescent β-subunit of PC was first synthesized artificially by *in vitro* autocatalysis. A group of lyase genes (*cpcS*, *cpcT*, *cpcU*, and *cpcV*) has been reported in *Synechococcus* sp. PCC 7002 (*Sy*7002) and the function of their encoded proteins analyzed. *cpeS* encodes the β82 lyase of PC in *Anabaena* sp. PCC 7120 and *cpcT* encodes the β153 lyase of PC in *Sy*7002 [25,26]. Both *CpcS* and *CpcU* are required for *in vitro* PCB synthesis in addition to the synthesis of β-PC of *Sy*7002 [27]. In addition, other highly homologous genes have been identified and characterized in other species of cyanobacteria [25].

In this study, we identified the *S6* genes *slr2049* and *sll0583*, which have high homology to *cpcS*, and characterized them as lyases that catalyze the linking between β82-PC of *Sp* and PCB. In addition, we analyzed the spectroscopy characteristics of several recombinant and natural PBPs. Although recombinant proteins might cause side effects in pharmacological use, such as allergy, their molecular design could provide more and better fluorescent tags for biotechnological applications.

1 Materials and methods

1.1 Generation of constructs

E. coli BL21 (DE3) and the genomic DNA of *Sp* and *S6* are maintained in our laboratory. The vectors pCDF (Merck KGaA, Darmstadt, Germany)-*ho1–pcyA*, and pCDF-*cpcB* were constructed, and recombinant holo-α-allophycocyanin (holo-α-APC) and holo-α-PC were expressed and purified in our laboratory. The primers used are shown in Table 1. Positions 153 and 82 of *cpcB* in pCDF-*cpcB* were mutated by site-directed mutagenesis using a MutanBEST Kit (TaKaRa, Tokyo, Japan). *Slr2049* was amplified from *S6* genomic DNA. The obtained vector pCDF-*ho1–pcyA* was digested by *Nde* I and *Xho* I to generate the *ho1–pcyA* fragment. Then, this fragment was ligated into *Nde* I and *Xho* I-digested pCDF-*cpcB*, pCDF-*cpcB* (C82I), and pCDF-*cpcB* (C153A). The PCR-amplified DNA fragment *slr2049* was purified and digested by *Sac* I and *Not* I, then ligated into *Sac* I and *Not* I-digested pCDF-*cpcB* (C82I)–*ho1–pcyA* and pCDF-*cpcB* (C153A)–*ho1–pcyA*. All vectors were transformed into competent *E. coli* BL21 cells using a standard procedure. The resulting colonies were selected on spectinomycin-containing media, screened by restriction enzyme digestion, and then confirmed by DNA sequencing.

1.2 Transformation and protein expression

All recombinant strains were streak-cultivated for 12 h. Then a single colony was chosen and grown in a shaking flask culture in 400 mL LB medium at 37°C until the A_{600} value was approximately 0.6. Then, isopropyl-β-*D*-thiogalactoside (IPTG) was added to the medium at 1 mmol L^{-1} and protein expression was induced by growing at 28°C for 6–8 h. Cells were centrifuged then washed twice with distilled water. Freshly prepared samples were stored at 4°C in the dark.

1.3 Purification of recombinant protein

The recombinant proteins constructed contained a 6-His tag at their N-terminus; thus they could be conveniently purified by HisTrap HP affinity Ni^{2+}-chelated chromatography (Amersham Biosciences, Uppsala, Sweden). Recombinant *E. coli* cell pellets were resuspended in binding buffer (20 mmol L^{-1} sodium phosphate, 0.5 mol L^{-1} NaCl, 20 mmol L^{-1} imidazole; pH 7.4) then disrupted by ultrasonication for 30 min (in alternating bursts with/without ultrasonication for 20 and 10 s, respectively). After centrifugation, the supernatant was loaded onto a pre-equilibrated 5-mL HisTrap HP Ni^{2+} affinity column (Amersham Biosciences), and the

Table 1 Primer sequences and PCR reaction conditions

Genes	Primers[a)]	Restriction enzymes	PCR recycle
cpcB	5′-TGTGTGGATCCGATGAAAACCCCCCTAAC-3′ 5′-ACAAGGAGCTCAACTAGCTTAGGGCGTTG-3′	*Bam*H I *Sac* I	30 cycles (94°C for 5 min, 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, 72°C for 10 min)
cpcB (C153A)	5′-GATGCAAGGGCTTTGGCTT-3′ 5′-GCCAGGAGTGATACCTGCG-3′		30 cycles (94°C for 30 s, 50°C for 30 s, 72°C for 5 min)
cpcB (C82I)	5′-ATGGCTGCTATCTTGCGTGAC-3′ 5′-ACGACGGCTGGTGTAGGC-3′		30 cycles (94°C for 30 s, 50°C for 30 s, 72°C for 5 min)
hoxI	5′-TGCATATGAGTGTCAACTTAGCTTCCCAG-3′ 5′-TTGATATCCTAGCCTTCGGAGGTGGCGAG-3′	*Nde* I *Eco*R V	30 cycles (94°C for 5 min, 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, 72°C for 10 min)
pcyA	5′-TTGATATCAATAAGGAGATATCACATGGCCGTCAC-TGATTTAAGTTTGACC-3′ 5′-TGCTCGAGTTATTGGATAACATCAAATAAGAC-3′	*Eco*R V *Xho* I	30 cycles (94°C for 5 min, 94°C for 1 min, 55°C for 1 min, 72°C for 1 min 30 s, 72°C for 10 min)
cpcS (*slr2049*)	5′-TAAGAGCTCATGCAATGGAATTCTT-3′ 5′-TAGTCGACCTACCAGCCACAAAAT-3′	*Sac* I *Sal* I	30 cycles (94°C for 5 min, 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, 72°C for 10 min)
cpcU (*sll0583*)	5′-ATTGTCGACATGAAGCCTGTTGCCCC-3′ 5′-ATAGCGGCCGCTTACTCTGCCGGAG-3′	*Sal* I *Not* I	30 cycles (94°C for 5 min, 94°C for 1 min, 59°C for 1 min, 72°C for 1 min, 72°C for 10 min)

a) The underlined sequences show the restriction enzyme recognition sequences.

sample was chromatographically separated by fast protein liquid chromatography at room temperature. After washing with 10 column volumes of binding buffer to remove the untagged proteins, the target protein was harvested with elution buffer (20 mmol L^{-1} sodium phosphate, 0.5 mol L^{-1} NaCl, 500 mmol L^{-1} imidazole; pH 7.4).

1.4 SDS-polyacrylamide gel electrophoresis (PAGE) and spectrometry

Protein samples were analyzed by 15% SDS-PAGE and Coomassie blue staining, and the chromophore-containing peptides were identified by Zn^{2+}-induced fluorescence and UV illumination [28]. Absorbance spectra were acquired on a computer-controlled DU650 UV-VIS spectrophotometer (Beckman Coulter, Fullerton, CA, USA). Fluorescence spectra were obtained using a F-4500 spectrofluorometer (Hitachi, Tokyo, Japan). The scan speed was 1000 nm min^{-1} and the slit width was 5.0 nm for all measurements.

2 Results

2.1 Recombinant protein expression, purification, and SDS-PAGE

We constructed vectors to express wild-type and mutant CpcB–*slr2049*–*sll0583*–*ho1*–*pcyA* (Figure 1).

(i) cpcB (C153A). After induction with IPTG, the culture containing pCDF-*cpcB* (C153A)–*slr2049*–*sll0583*–*ho1*–*pcyA* looked blue (Figure 2). Visualization of purified protein on a Coomassie blue-stained SDS-polyacrylamide gel showed one distinct band at approximately 20.0 kD (Figure 3, lane B), corresponding to the calculated molecular mass of

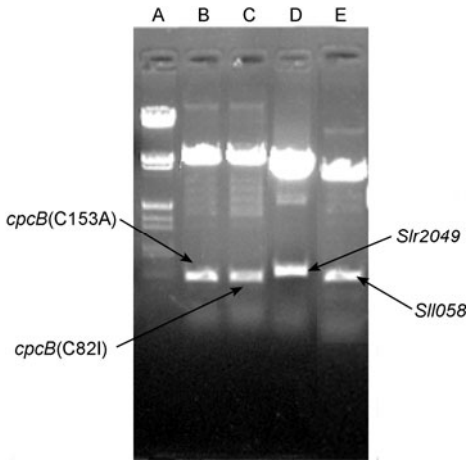

Figure 1 Restriction enzyme digestion of constructs. A, Lambda DNA/*Eco*R I+*Hin*d III marker; B, pCDF-*cpcB* (C153A)–*slr2049*–*sll0583*–*ho1*–*pcyA* digested with *Bam*H I and *Sac* I; C, pCDF-*cpcB* (C82I)–*slr2049*–*sll0583*–*ho1*–*pcyA* digested with *Bam*H I and *Sac* I; D, pCDF-*cpcB* (C82I)–*slr2049*–*sll0583*–*ho1*–*pcyA* digested with *Sac* I and *Sal* I; E, pCDF-*cpcB* (C82I)–*slr2049*–*sll0583*–*ho1*–*pcyA* digested with *Sal* I and *Not* I.

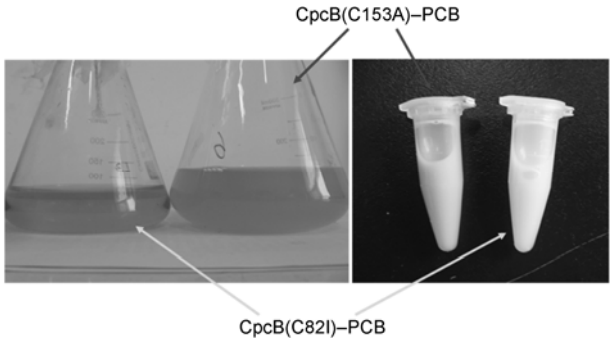

Figure 2 Expression of recombinant proteins after induction with IPTG for 6–8 h. CpcB (C153A)–PCB was produced from the construct pCDF-*cpcB* (C153A)–*slr2049*–*sll0583*–*ho1*–*pcyA*. CpcB (C82I)–PCB was produced from the construct pCDF-*cpcB* (C82I)–*slr2049*–*sll0583*–*ho1*–*pcyA*.

CpcB (C153A). Covalent binding of the chromophore was confirmed by exposure to Zn^{2+} and UV illumination, upon which an orange fluorescence was emitted (Figure 3, lane b).

(ii) cpcB (C82I). The culture containing pCDF-*cpcB* (C82I)–*slr2049*–*sll0583*–*ho1*–*pcyA* did not show any color change after induction for 6–8 h (Figure 2). A distinct CpcB (C82I) band at approximately 20.0 kD (Figure 3, lane E) was observed, but no chromophorylated protein was detected by Zn^{2+} staining and UV illumination (Figure 3, lane e).

2.2 Spectral analysis

(i) cpcB (C153A). The absorbance spectrum of *Sp* cpcB (C153A)–PCB had a λ_{max} of 624 nm, ε_M=205000 M^{-1} cm^{-1}, and an $A_{624\ nm}$:$A_{369\ nm}$ ratio of 4.97 in elution buffer (Figure 4). The maximum fluorescent emission peak of *Sp* cpcB

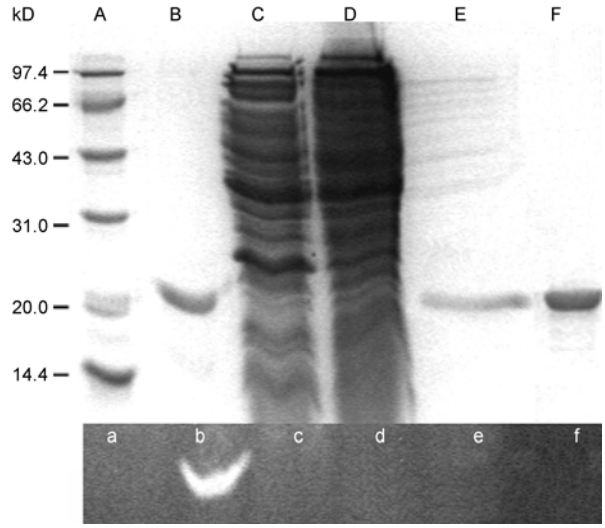

Figure 3 SDS-PAGE and Zn^{2+} staining of expressed proteins. A, Standard protein marker; B, purified CpcB (C153A)–PCB; C, proteins of *E. coli* BL21 transformant containing vector pCDF induced for 6 h; D, proteins of *E. coli* BL21 induced for 6 h; E, purified CpcB (C82I); F, purified CpcB. a–e, UV illumination of Zn^{2+}-stained chromoproteins in the corresponding lanes A–E.

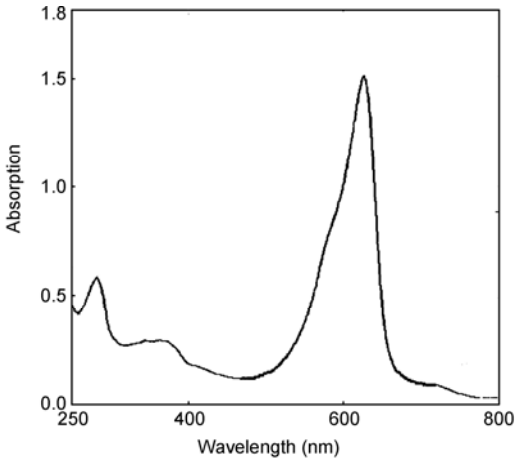

Figure 4 Absorption spectrum of recombinant CpcB (C153A).

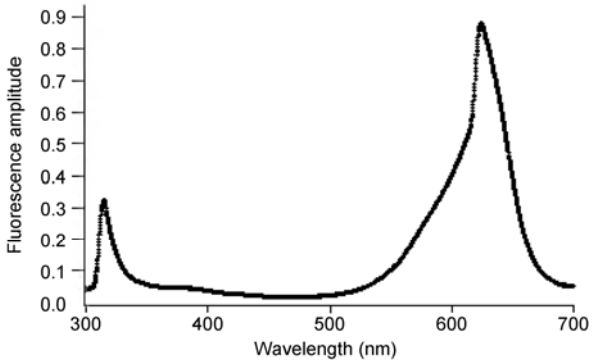

Figure 5 Emission spectrum of recombinant CpcB (C153A) under 580-nm excitation.

(C153A)–PCB was 645 nm under 580-nm excitation (Figure 5).

(ii) cpcB (C82I). No characteristic peaks were found in either the absorption or the fluorescent spectrum. The absorption λ_{max} of native APC, PC, holo-α-PC, and holo-α–APC expressed in our laboratory was 652, 625, 621, and 623 nm, respectively (Table 2; Figures 6 and 7). Their fluorescence emission λ_{max} values are shown in Table 2.

Table 2 Spectra of *Sp* native APC and PC and of several recombinant PBPs

Spectrum	Native		Recombinant		
	APC	PC	Holo-α-PC	CpcB(C153A)–PCB	Holo-α–APC
Abs (λ_{max}, nm)	652	625	621	624	623
Em (λ_{max}, nm)	660	643	644	645	647

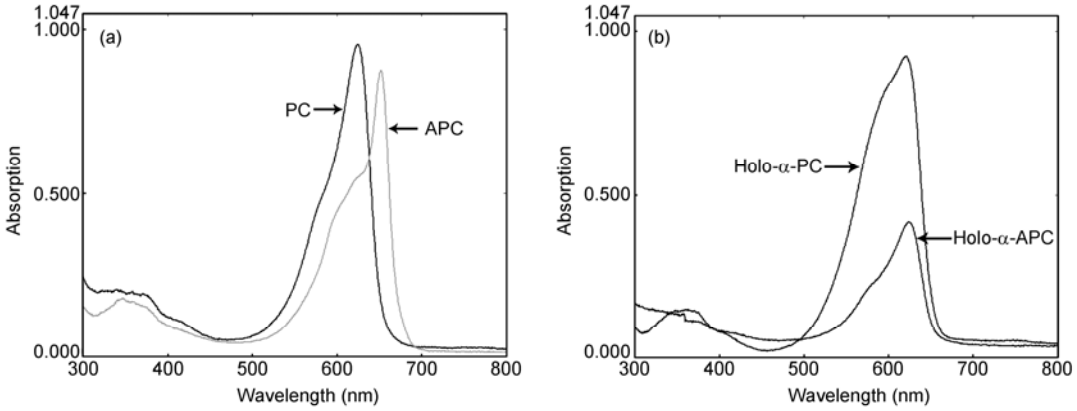

Figure 6 Absorption spectra of native APC and PC (a) and recombinant holo-α-PC and holo-α-APC (b).

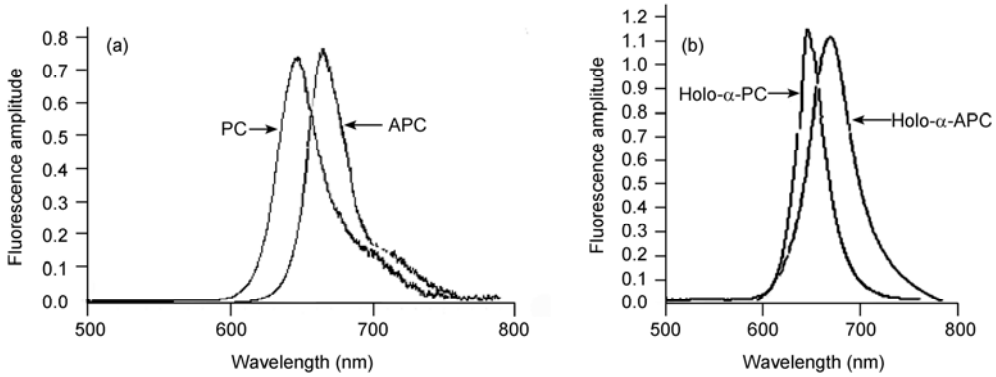

Figure 7 Emission spectra of native APC and PC (a) and recombinant holo-α-PC and holo-α-APC under 580-nm excitation (b).

3 Discussion

Previously, we successfully expressed fluorescent *S6* holo-α-PC using the construct pCDF-*cpcA–cpcE–cpcF–ho1–pcyA* [24]. In this study, we constructed pCDF-*cpcB* (C153A)–*slr2049–sll0583–ho1–pcyA*, and confirmed that the predicted lyase genes *slr2049* and *sll0583* did exhibit bilin lyase activity *in vivo*. The reaction catalyzed by the *S6* bilin lyases—linking *Sp* apo-β-PC to *E. coli* PCB—was achieved, as evidenced by the production of recombinant of CpcB (C153A)–PCB that was fluorescent upon Zn^{2+} staining and UV illumination. Furthermore, the obtained CpcB (C153A)-PCB could be conveniently purified because of its His tag.

Interestingly, the lyases encoded by *slr2049* and *sll0583* were able to catalyze linkage of PCB at the Cys at position 82β of CpcB (C153A), but not at the Cys at position 153β of CpcB (C82I). This is unlikely to be because the mutation disrupted the protein structure, because the Cys at the chromophore-binding site was mutated to the neutral amino acid Ala that contains an amino radical and a carboxylic radical; thus, the structure of the mutant was probably unchanged compared with that of the wild type [29].

We have demonstrated an effective approach to the generation of α- and β-subunits of PBPs, even holo-PBPs, and have contributed to the understanding of the natural assembly mechanism of PBSs by recombinant PBPs *in vivo*.

We revealed differences in the spectral characteristics between recombinant and native PBPs. For instance, the UV-Vis absorption spectrum λ_{max} of recombinant holo-α-PC and CpcB (C153A)–PCB showed 4- and 1-nm shifts, respectively, compared with the native proteins. Furthermore, the fluorescence emission spectrum λ_{max} of recombinant holo-α-PC and CpcB (C153A)–PCB demonstrated 1- and 2-nm shifts, respectively, compared with the native proteins. Another distinction is that the UV-VIS absorption spectrum λ_{max} of autocatalyzed recombinant holo-α-APC showed a 29-nm difference from that of native APC, while the fluorescence emission λ_{max} of autocatalyzed recombinant holo-α-APC demonstrated a 13-nm shift compared with native APC. These differences may occur for the following reasons. First, the lyase functions to stabilize chromophore conformation [30]. However, the spontaneous linking of the chromophore to apo-α-APC in recombinant holo-α-APC can lead to conformational change [31]. Second, the λ_{max} shift of the holo-α-APC fluorescent spectrum is larger than that of the holo-α-PC absorption spectrum. The precise arrangement of the chromophore and its surrounding residues may cause a time delay between the absorption change and the fluorescence change in the course of chromophore assembly [5]. The linking reaction between the chromophore and apo-α-APC is probably a biphasic reaction in which the fluorescence signals might not reach it when the absorption signals approach saturation. The spectroscopic findings of recombinant holo-α-PC and CpcB (C153A)–PCB were similar to those of native PC, while recombinant holo-α-APC deviated from native APC. Therefore, recombinant subunits of PC may be effectively expressed by gene engineering, thus showing high potential for useful application. Finally, we expected to produce the monomer structure of recombinant protein, but it may have aggregated into multimers during purification and storage. The λ_{max} of the recombinant PC protein approached the λ_{max} of the monomer, but shifted to the λ_{max} of the trimer. Thus, steps should be taken to avoid aggregation and to retain the monomer structure.

The homologs of the CpcS and CpeS proteins can be classified into five groups [27]. In *Nostoc* sp. PCC 7120, a single CpeS-like protein, Alr0617, can catalyze the attachment of PCB to Cys-82 of β-PC and β-phycoerythrocyanin as well as several AP subunits [32]. CpcS-I and CpcU function together as phycobilin lyases in the attachment of the chromophore at Cys-82 for β-PBPs in *Sy*7002; acting alone, neither gene product can attach the PCB chromophore to this binding site [27]. CpcS-I and CpcU from *S6* are also required for the addition of PCB to β-PC of this strain *in vitro* [33]. These observations are consistent with data from phylogenetic analyses based on sequence alignment [26].

Some of the most important uses for fluorescence are in immunology, in fluorescent tags, and in biomedical research. The natural PBPs in *Sp* have several biological activities. It has been shown that recombinant APC has antitumor activity whereas fluorescent recombinant holo-α-PC and holo-α-APC have antioxidant activity [34,35]. Gene-engineering techniques can help express PBPs effectively and with a stable structure, to facilitate their application in multiple fields.

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