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Combinational Biosynthesis of a Fluorescent Cyanobacterial Holo-α-Phycocyanin in *Escherichia coli* by Using One Expression Vector

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Abstract The phycobiliproteins (PBSs) are large pigment proteins found in certain algae that play a central role in harvesting light energy for photosynthesis. Phycocyanin (PC) is one type of PBSs that gains increasing attention owing to its various biological and pharmacological properties. In this paper, an expression vector containing five essential genes in charge of biosynthesis of cyanobacterial C-phycocyanin (C-PC) holo-α subunit (holo-CpcA) was successfully constructed resulting in over-expression of a fluorescent holo-CpcA in *E. coli* BL21. The vector harbored two cassettes: one cassette carried genes *hox1* and *pcyA* required for conversion of heme to phycocyanobilin (PCB), and the other cassette carried *cpcA* encoding CpcA along with *cpcE* and *cpcF* both of which were necessary and sufficient for the correct addition of PCB to CpcA. The vector system contained a His-tag for protein purification. The purified protein showed correct molecular weight on SDS-PAGE gel and emitted orange fluorescence by UV excitation. The maximum peak of absorbance spectrum was at 623 nm, and the maximum peak of fluorescence emission and excitation were at 648 and 633 nm, respectively, which were similar to those of native C-PC. This study provides an efficient method for large-scale production of the fluorescent holo-CpcA in biotechnological applications.

Keywords *Escherichia coli* · Phycocyanin · Fluorescence · Holo-HT-CpcA

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Abbreviations

PBS	phycobiliproteins
CpcA	α subunit of C-phycocyanin
CpcE and CpcF	subunits of the heterodimeric phycocyanin α-subunit phycocyanobilin lyase
Hox1	heme oxygenase 1
HT	His-tag
PCB	phycocyanobilin
PcyA	3*Z*-phycocyanobilin: ferredoxin oxidoreductase
Sp	spectinomycin
Km	kanamycin

Introduction

Phycobiliproteins are a family of light-harvesting proteins found in cyanobacteria, red algae, and the cryptomonads and are highly organized complexes composed of various biliproteins and linker polypeptides [1, 2]. PBSs are classified into four types based on their spectrum properties: phycoerythrin (PE), phycoerythrocyanin (PEC), phycocyanin (PC) and allophycocyanin (APC) [2–4]. They function as predominant light harvesting complexes to absorb the light from 480 to 660 nm and efficiently transfer the light energy to chlorophyll *a* [5]. They are normally composed of α and β subunits existing in trimer $(\alpha\beta)_3$ or hexamer $(\alpha\beta)_6$ made up of equimolar monomer (αβ) [6].

PC is a species of water soluble, nontoxic fluorescent protein with potent antioxidant, anti-inflammatory and anticancer properties [7]. There were widespread applications of PCs as fluorescent tags *in vitro* and *in vivo* [8–10]. Hence, the biotechnological production of fluorescent PCs at large scale is looked forward to for both research and application purposes.

Today, the biosynthetical pathway of C-PC holo-α subunit in *Synechocystis* sp. PCC6803 has been elucidated [11]. Totally five genes were necessary: i.e., *cpcA* encoding C-phycocyanin α subunit (CpcA); *cpcE* and *cpcF* in charge of correct addition of PCB to CpcA; *hox1* and *pcyA* encoding enzymes necessary for conversion of heme to PCB.

Tooley et al. [11] reported expression of five genes in *E. coli* by using two expression vectors to produce holo-CpcA, which may lead to expression instability of all the five foreign genes. This research reports a new combinational biosynthesis strategy in *E. coli* by using one expression vector containing all the necessary five genes (*hox1*, *pcyA*, *cpcA*, *cpcE*, and *cpcF*) and a His-tag for convenient purification of recombinant protein. The result may provide a fast and efficient way of production of fluorescent holo-HT-phycocyanin at large scale Fig. 1.

Materials and Methods

DNA Extraction and PCR Amplification

For total DNA isolation from *Synechocystis* sp. PCC6803, A Universal Genomic DNA Extraction Kit Ver.3.0 (TaKaRa, Japan) was used according to the manufacturer's

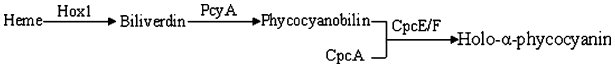

Fig. 1 The biosynthetic pathway of holo_α_ phycocyanin from heme and CpcA

instructions. To produce holo-HT-CpcA in *E. coli*, five genes were amplified from *S.* sp. PCC6803 genome DNA by using polymerase chain reaction (PCR). The gene sequence data for primer device was obtained from GenBank (accession no. BA000022). Primer sequences and PCR parameters were described in Table 1. All primers used in this paper were synthesized by Sangon Co., Ltd. (China). After PCR, sequencing was carried out to check the integrality of amplified genes.

Construction of the Expression Vector

PET28a+ (Novagen, Germany) was the starting vector used to construct the 1.99 kb cassette *cpcA-cpcE-cpcF* by cloning into 0.49 kb *Bam*HI–*Sac*I digested amplified *cpcA*, 0.88 kb *Sac*I–*Sal*I digested *cpcE*, and 0.62-kb *Sal*I–*Not*I digested *cpcF*, respectively (Fig. 2). pCDFDuet™-1 (pCDF, Novagen, Germany) was another starting vector containing a *his-tag*, two expression cassettes and two multiple cloning sites (MCS) each of which was initiated by a T7*lac* promoter and followed by a ribosome binding site (*rbs*). pCDFDuet™-1 also carries the CloDF13-derived CDF replicon, *lacI* gene and a spectinomycin resistance gene (*Sp*) (Fig. 2). To construct the expression vector pCDF-*cpcA-cpcE-cpcF-hox1-pcyA* for *E. coli* BL21, the *cpcA-cpcE-cpcF* cassette was cut off from pET28a+-*cpcA-cpcE-cpcF* using *Not*I and *Bam*HI, and then cloned into the first expression cassette of *Not*I-*Bam*HI digested pCDF. The second expression cassette of pCDF was used

Table 1 Information of genes correlated to biosynthesis of holo-α-phycocyanin with His-tag.

Genes	Primers	Restriction enzymes	PCR recycle
hoxI	5′TG*CATATG*AGTGTCA ACTTAGCTTCCCAG3′	*Nde* I	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)
	5′TT*GATATC*CTAGCCTTCG GAGGTGGCGAG3′	*Eco*R V	
pcyA	5′TT*GATATC*AATAAGGAGATAT CACATGGCCGTC ACTGATTTAAGTTTGACC3′	*Eco*R V	30 cycles (94°C for 5 min, 94°C for 1 min, 55°C for 1 min, 72°C for 1 min30s, 72°C for 10 min)
	5′TG*CTCGAG*TTATTGGATAACATC AAATAAGAC3′	*Xho* I	
cpcA	5′TGTGT*GGATCC*GATGAAAACC CCCCTA AC3′	*Bam*H 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)
	5′ACAAG*GAGCTC*AACTAGCTT AGGGCGTTG3′	*Sac* I	
cpcE	5′GTA*GAGCTC*AAGGAGATATAC CATGCAGGATTC TGAATCAAAAAC3′	*Sal* I	30 cycles (94°C for 5 min, 94°C for 1 min, 50°C for 1 min, 72°C for 1 min30s, 72°C for 10 min)
	5′AGC*GTCGAC*GTTCATAATAGAGA ATCCATCAG3′	*Not* I	
cpcF	5′CGC*GTCGAC*AAGGAGATATACCA TGACAAAGGT TGAGGAACTAATTTTAG3′	*Sal* I	30 cycles (94°C for 5 min, 94°C for 1 min, 55°C for 1 min, 72°C for 1 min30s, 72°C for 10 min)
	5′AATAAGCTT*GCGGCCGC*TCATC CTAGCCAGACT AGCC3′	*Not* I	

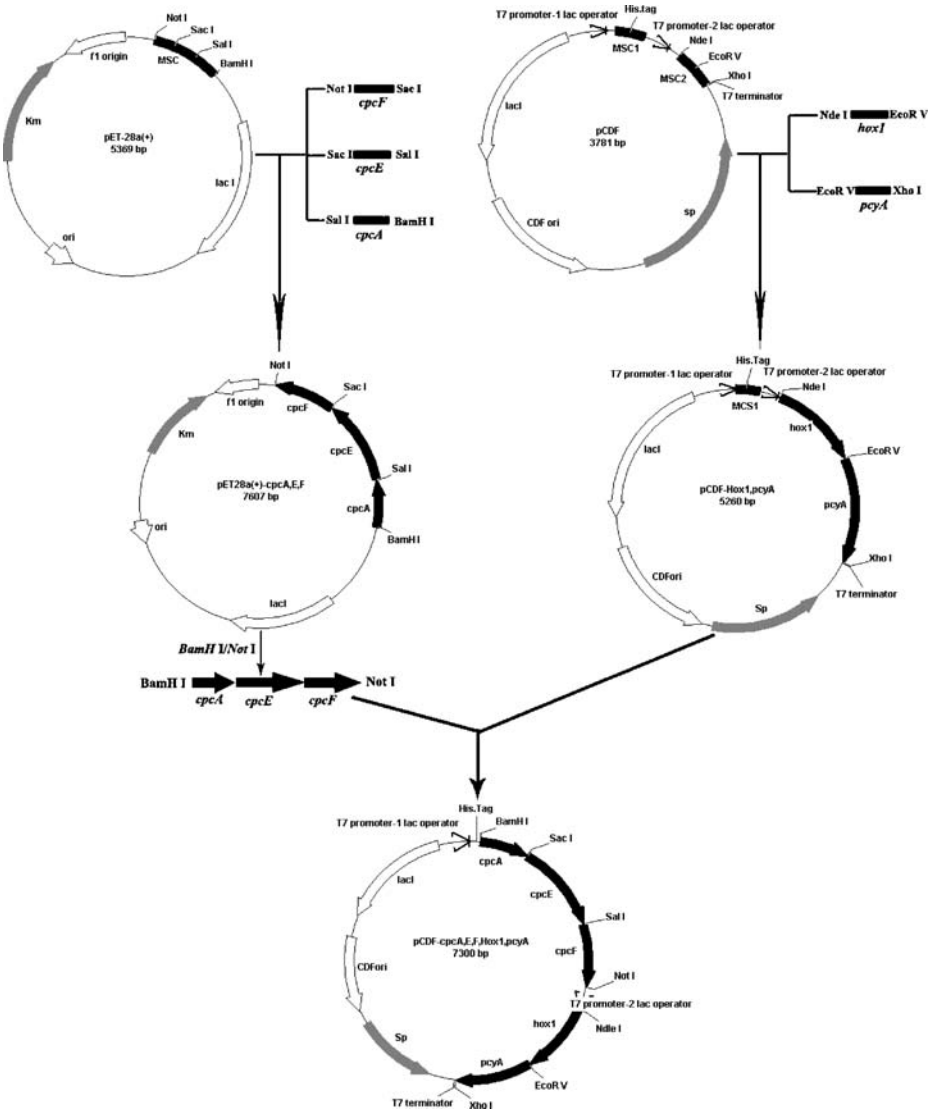

Fig. 2 Flow chart of expression vector construction

to insert into the 0.72 kb *Ned*I–*Eco*RV digested *Hox1* and the 0.75 kb *Eco*RV–*Xho*I digested *pcyA*, successively.

To ensure the validity of the targeted gene in each step, every transformant was screened with both restriction enzymatic digestion (BRED) analysis and then confirmed by DNA sequencing. All amplified sequences were blasted with the reported sequences in NCBI. The transformants transformed with pET28a+ were selected by using kanamycin, and those transformed with pCDF were selected by using spectinomycin (Sp), respectively. All the vectors were transformed into the competent *E. coli* BL21 cells via a standard procedure [12].

Induction of Expression and Purification of Recombinant Proteins

The pCDF-*cpcA-cpcE-cpcF-hox1-pcyA* expression vector was finally transformed into *E. coli* BL21. An overnight pre-culture of the positive transformant was made in 400 ml Luria–Bertani (LB) medium till OD_{600} reached 0.8–1.0. The culture was then cooled down to 28°C before induction with 0.1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) for further 6 h. The bacteria were harvested by centrifugation and washed twice with sterile distilled water at 4°C in a J2-HS Centrifuge (Beckman, US) at 10,000×*g* for 10 min.

The supernatant was discarded, and cells were thawed at room temperature in sodium phosphate buffer (20 mM, pH 7.0) and then sonicated under 1,100 psi for 15 min at 0°C with 20 s treatment/10 s interval using a Ultrsonic Processor CP 130 (Cole-Parmer Instruments, US). Cell debris was removed by centrifugation at 4°C in a Biofuge 28 RS centrifuge (Heraeus Speatech, Germany) at 10,000×*g* for 15 min. Protein purification procedure using Chelating Sepharose Fast Flow Column (Amersham Biosciences, Sweden) was carried out at 4°C according to the manufacturers' instructions by using Ni^{2+} as the transition metal ions. The column was washed with the following solutions in turn: 5× column volumes of distilled water, 2× column volumes of metal ion solution (0.1 M $NiCl_2$), 5× column volumes of distilled water, and finally, 3× column volumes of start buffer (20 mM potassium phosphate, pH 7.0, 0.5 M NaCl) provided by the manufacturer. The supernatant was then loaded directly onto Ni^{2+}-treated chelating affinity column. Untagged proteins were washed off using start buffer and then elution buffer (20 mM potassium phosphate, pH 6.0, 0.5 M NaCl). Holo-HT-CpcA was finally eluted using start buffer supplied with 50 mM imidazole.

SDS-PAGE, Western-Blot, and Spectrometry Analysis

The purity of above purified His-tagged protein was analyzed on 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Chromophore-containing peptides were identified under Zn^{2+}-induced fluorescence before Coomassie staining. The gels were soaked in 1 M $ZnSO_4$ solution for 1 h before visualization under UV light [13].

Western-blot analysis was performed according to Ge et al. [14]. Polyclonal rabbit anti-PBSs antibody was used as primary antibody, and horseradish peroxidase labeled affinity-purified goat anti-rabbit IgG was used as the secondary antibody.

Absorbance spectra were acquired on a computer-controlled UV- Spectrophotometer DU650 (Beckman, US). Corrected fluorescence spectra were obtained with F-4500 Spectrofluorometer (HITACHI, Japan). The excitation and emission slits were set at 5 nm for all measurements.

Results

Construction of the Expression Vector

Five genes were successfully amplified and ligated into the prokaryotic expression vector pCDF as planned. Five vectors (pCDF- *hox1*; pCDF-*hox1-pcyA*; pET28a+-*cpcA*; pET28a+-*cpcA-cpcE*; pET28a+-*cpcA-cpcE-cpcF*) were constructed other than the final expression vector pCDF-*cpcA-cpcE-cpcF-hox1-pcyA*, which harbored CloDF13-derived CDF replicon, *lacI* operator sequence for tight control of expression under control of the T7 promoter, two cassettes carrying *cpcA*, *cpcE*, *cpcF* and *pcyA*, *hox1*, respectively, and a His-tag for further

purification. All the vectors showed correct restriction enzyme pattern in BRED analysis. Sequencing data showed correct ORFs of *cpcA*, *cpcE*, *cpcF*, *Hox1*, and *pcyA* in pCDF in correct orientation.

Induced Expression and Protein Purification

After induction with IPTG, the transformants exhibited a blue color, whereas the blank *E. coli* culture did not give any color change. Visualization of purified protein on Coomassie blue-stained SDS-PAGE gel, showed only one distinct band of 21.1 kDa (Fig. 3, lane a), corresponding to the calculated molecular masses of holo-HT-CpcA. Covalent binding of the chromophore was confirmed upon exposure to Zn^{2+} and UV illumination in which an orange fluorescence emitted (Fig. 3, lane f). Furthermore, Western blot analysis confirmed the identity of holo-HT-CpcA and no degradation fragment was detected (Fig. 3, lane g).

Characterization of the Absorbance Spectrum

The absorbance spectrum of holo-HT-CpcA had a λ_{max} at 623 nm with εM=109,000 M^{-1} cm^{-1} and the ratio of $A_{623\ nm}$: $A_{369\ nm}$ is 6.41 (Fig. 4). The previous reported λ_{max} for recombinant *S.* sp. PCC6803 holo-HT-CpcA is at 625 nm, and the ratio of $A_{625\ nm}$: $A_{369\ nm}$ is 4.14 [11]. The corresponding value for native *Sy.* sp. PCC6803 holo-CpcA is 620 nm and 4.42, respectively. The absorbance properties of other native holo-CpcA from cyanobacteria are at similar values [4].

Characterization of the Fluorescence Spectrum

The fluorescence emission λ_{max} of the holo-HT-CpcA in this research was at 644 nm (Fig. 4), compared with 642 nm in previous reported *S.* sp. PCC6803 holo-CpcA [11]. The excitation λ_{max} of the holo-HT-CpcA in this research was at 633 nm compared with 625 nm in previous reported *S.* sp. PCC6803 holo-CpcA (11; Fig. 5).

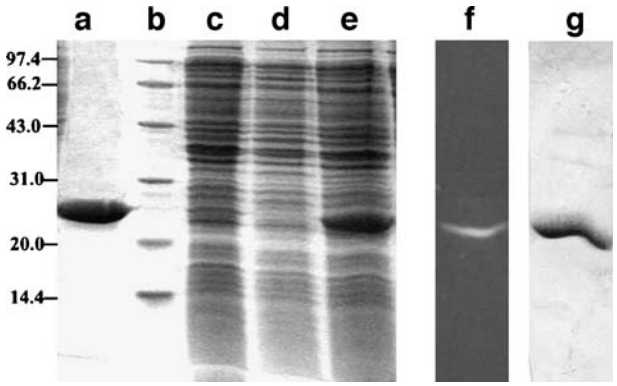

Fig. 3 Analysis of expressed and purified fusion proteins by gradient SDS_PAGE (15%) and Western_blot. *lane a*, purified holo-HT- CpcA; *lane b*, protein marker; the protein bands correspond to 97.4, 66.2, 43, 31, 20, and 14.4 kDa (from *top to bottom*); *lane c*, proteins of *E. coli* BL21; *lane d*, proteins of *E .coli* BL21 with plasmid pCDF; *lane e*, proteins of plasmid pCDF-*cpcA-cpcE-cpcF-hox1-pcyA* in BL21; *lane f*, proteins visualized by UV-excitation in the presence of Zn^{2+}; *lane g*, Western blotting analysis of recombinant holo-HT-CpcA with polyclonal rabbit anti-PBS antibody as the primary antibody and HRP-labeled goat anti-rabbit antibody as the secondary antibody

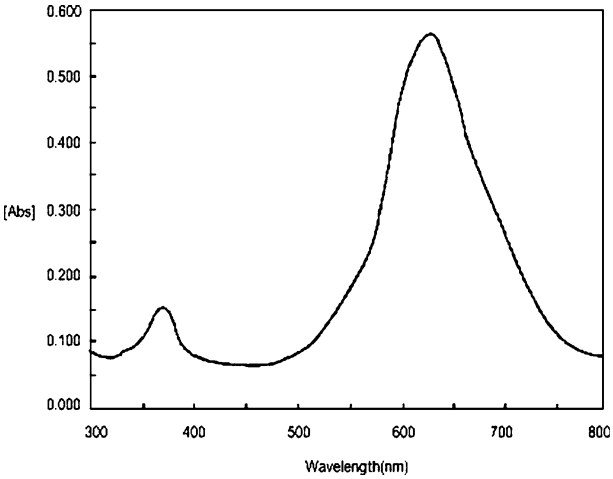

Fig. 4 Absorption spectrum of affinity-purified holo-HT-CpcA expressed in *E.coli* (Amax623)

Discussion

PC is brilliantly colored and water-soluble, and these features make it an ideal candidate of fluorescent labels in practice [7, 10]. This research presents evidence of new strategy of expression and purification of florescent holo-CpcA by using one expression vector. This expression vector contains five genes in two cassettes (Fig. 2): the phycocyanin alpha subunit, *cpcA*, two genes required for the bilin attachment, *cpcE* and *cpcF*, *hox1* and *pcyA* required for the conversion of heme to PCB. Tooley et al. (2001) biosynthesized C-phycocyanin holo-α subunit by using two vectors: pBS414V (*ht-cpcA-cpcE-cpcF* cassette, Sp^r) and pAT101 (*ht-hox1-pcyA* cassette, Km^r). In our work, the stability of transformants are increased due to using one expressed vector and selecting only by one resistant marker. The present method is time saving, good for large-scale production of recombinant holo-CpcA for further research and application. SDS-PAGE gel (Fig. 3) showed that there is one

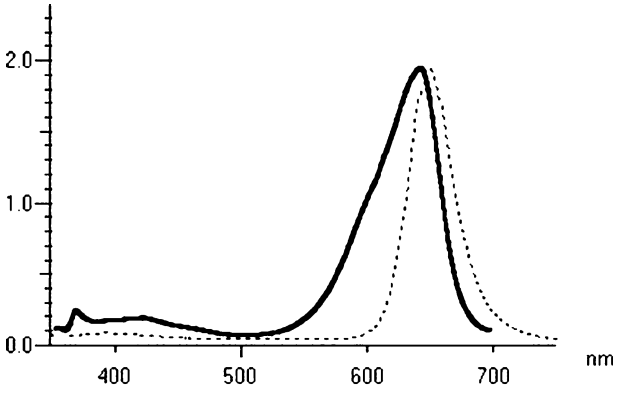

Fig. 5 Fluorescence emission (λ_{max} 644 nm, *thin solid line*), and fluorescence excitation (λem 633 nm, *dashed line*) recorded from 600 to 800 nm (excitation and emission slits were 5 nm). All spectra were measured at room temperature. The fluorescence excitation spectrum was normalized to the absorbance spectrum at 625 nm

band (holo-HT-CpcA) at 21.1 kDa, while Tooley's work yielded two separate bands holo- and apo-HT-CpcA [11].

The absorption spectrum of holo-HT-CpcA in this research is similar to native CpcA, but there is a 2-nm difference. Compared with native holo-CpcA, the fluorescence spectrum of holo-HT-CpcA showed red shift: 9 nm in excitation spectrum and 2 nm in emission spectrum. The reason may lie in the fact that CpcE /F could lead to the deflection of spectrum [15].

Holo-CpcA has a special spectroscopic property that is superior to many organic fluorescent dyes [10], which can be used in medical tagging and combination therapy. Vector strategy in this research can be also applied in combinational biosynthesis of other types of holo-α and β subunits of PBSs, which benefits co-expression multi-genes and convenient purification using His-tag.

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