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Highly soluble and stable recombinant holo-phycocyanin alpha subunit expressed in *Escherichia coli*

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ABSTRACT

C-phycocyanin (Cpc) is one of the phycobiliproteins with highly fluorescent and various pharmacological activities. Holo-Cpc-α subunit (holo-CpcA) expressed in *Escherichia coli* resulted in low yield and tended to aggregate after purification. In this study, we constructed a new plasmid coding holo-CpcA fused with hexahistidine and maltose-binding protein tag, which designated as HMCpcA, to improve its solubility and stability without the impairment of its spectra and fluorescent properties. HMCpcA was significantly more stable over time and a wider range of pH as compared to holo-CpcA. In addition, both the solubility and yields of HMCpcA increase significantly. We here provided an example to demonstrate that MBP could also improve the stability of the protein it fused while it has been reported as a soluble fusion partner before. This novel fluorescent protein will facilitate the large-scale production and be potentially applicable for the development of fluorescent probes, as well as antioxidant agents.

1. Introduction

Phycobiliproteins are light-harvesting antenna pigments found in cyanobacteria, red algae and the cryptomonds [1–4]. C-phycocyanin (Cpc), allophycocyanin (Apc), and phycoerythrocyanin (Pec) are the three most common phycobiliproteins in cyanobacteria. They are highly fluorescent due to the particular linear tetrapyrrole bilins covalently attached to the proteins and have been widely used as fluorescent probes [5,6]. Recently, phycobiliproteins have also been reported to have many pharmacological activities, among which the antioxidant and radical scavenging are well documented [7–9].

Currently, phycobiliproteins used in these researches are mainly extracted from algae, however, recombinant phycobiliproteins are also reported and proved to have antioxidant and anti-cancer properties [10–13]. Recombinant proteins expressed in *Escherichia coli* (*E. coli*) provide an attractive way in large-scale production. The extensive studies of the entire pathway of the biosynthesis of phycocyanobilin (PCB) and its addition to Cpc-α subunit (CpcA) has facilitated the protein engineering of holo-CpcA [14–21]. PCB derived from heme is catalyzed by heme oxygenase (Hox1) and further reduced by bilin reductase (PcyA). It is possible to produce bilins in *E. coli* as it can naturally synthesize heme. And the PCB heterodimeric lyase (CpcE and CpcF) is necessary for the combination of PCB to CPC-α subunit [17].

This entire pathway of holo-CpcA has been reconstituted in *E. coli* in our lab and other labs elsewhere [22–25]. However, the yields of recombinant holo-CpcA were only about 0.4 mg/g wet weight of *E. coli* cell [24], possibly due to the formation of inclusion body. In addition, the recombinant holo-CpcA tends to aggregate during the process of purification [10,14].

Maltose-binding protein (MBP) is derived from *E. coli* and has been proven to enhance the solubility and promote the proper folding of the attached protein, while His-tag can facilitate the purification. Therefore, the strategy combining a dual His6-maltose-binding protein (HisMBP) affinity tag appears to exploit the unique benefits of each tag and has become a popular method to achieve heterologous proteins of interest [26,27].

Phycoerythrin apo-subunits had been reported to fuse with MBP to improve their solubility expressed in *E. coli* previously [33]. As phycoerythrin apo-subunits can bind chromophores autocatalytically, highly fluorescent proteins were formed both in vitro and in vivo when incubated with exogenous phycoerythrobilin. Here

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Table 1
List of primers synthesized for plasmid vector construction.

Genes	Primers	Restriction enzymes
cpcA	5′-ACGGATCCGATGAAAACCCCTTTAAC-3′	*Bam*H I
	5′-GCGAGCTCCTAGCTCAGAGCATTGA-3′	*Sac* I
cpcE	5′-GTAGAGCTCAAGGAGATATACCATGAGTGAACCTAACCTCAAC-3′	*Sac* I
	5′-AGCGTCGACTCAGAGTAAACTATCCA-3′	*Sal* I
cpcF	5′-CGCGTCGACAAGGAGATATACCATGGAGGGTAATAGCGTC-3′	*Sal* I
	5′-ATAAGCTTGCGGCCGCCTAGATTGGGCCGATGT-3′	*Not* I
*hox*1	5′-ACGCATATGAGTGTCAACTTAGCTTCC-3′	*Nde* I
	5′-ATTGATATCCTAGCCTTCGGAGGTGGC-3′	*Eco*R V
pcyA	5′-TAGATATCAATAAGGAGATATACCATGGCCGTCACTGATTTAAG-3′	*Eco*R V
	5′-TGCTCGAGTTATTGGATAACATCAAAT-3′	*Xho* I
mbp	5′-GCAGGATCCGATGAAAATCGAAGAAGGTAAAC-3′	*Bam*H I
	5′-ATTGGATCCTCGATCCCGAGGTTGTT-3′	*Bam*H I

The restriction enzyme sites were underlined.

we reported a method combining a dual HisMBP affinity tag to improve the solubility and stability of holo-CpcA without the external addition of chromophore. This novel fluorescent protein will be potentially applicable for the development of fluorescent probes, as well as antioxidant agents.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

BL21 (DE3) was used for expression host. Plasmid pCDF Duet^{TM-1} (Novagen, Germany) containing two T7 lac promoters, was used to construct and express holo-CpcA with his6 tag and with HisMBP tag, which designated as HCpcA and HMCpcA, respectively. Cultures were grown in LB medium (comprising 10 g/L peptone, 5 g/L yeast extract and 10 g/L NaCl, pH 7.0) containing 100 mg/L spectinomycin (Sp).

2.2. Construction of expression vectors

In general, standard procedures were used for DNA manipulation. Full length *cpcA*, *hox1*, *pcyA*, *cpcE* and *cpcF* genes from *Synechocystis* sp. PCC6803 and MBP gene from the plasmid pMAL-p2X (New England Biolabs) were amplified by PCR using specific primers (Table 1). The amplified fragments were digested with the appropriate restriction enzymes. To express HCpcA, the amplified fragments were ligated into the pCDFDeut-1 vector, with *cpcA*, *cpcE* and *cpcF* in one cassette and *hox1*, *pcyA* in the other cassette. The resultant plasmid was designated as pHPC (Fig. 1). To express HMCpcA, the MBP amplified fragment was digested with *Bam*HI and then inserted in the unique *Bam*HI site of the plasmid pHPC. The resultant plasmid, named pHMPC, has a dual HisMBP affinity tag (Fig. 1). All the constructed plasmids were sequenced to check the validity of the gene sequences.

2.3. Purification of the recombinant proteins

The recombinant plasmids described above were individually transformed into *E. coli* BL21(DE3). The bacteria were cultured in LB medium with shaking at 37 °C. After an absorbance of 0.8 measured at 600 nm was reached, the culture temperature was reduced to 25 °C and isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 0.2 mM to induce expression for 12 h. The cultures were harvested by centrifugation at 5000 × *g* for 10 min at 4 °C. Cell pellets were then resuspended in 100 ml buffer A (20 mM sodium phosphate and 500 mM NaCl, pH 7.4). After sonication, the cell debris was removed by centrifugation at 10,000 × *g* for 30 min at 4 °C. The supernatant was loaded onto an immobilized metal affinity column (IMAC) of Chelating Sepharose (GE Healthcare Bio-Sciences) charged with Ni^{2+}, which had been pre-equilibrated with Buffer A.

For purification of HMCpcA, the column was washed with 5 column volumes of Buffer B (Buffer A with 50 mM imidazole, pH 7.4) to remove the unbound proteins and then the protein was eluted with 2 column volumes of Buffer C (Buffer A with 100 mM imidazole, pH 7.4). The pooled fractions were dialyzed against water and then freeze-dried.

For purification of HCpcA, the column was washed with 5 column volumes of Buffer B and then the protein was eluted with 2 column volumes of Buffer D (Buffer A with 300 mM imidazole, pH 7.4). The pooled fractions were then loaded onto a Superdex 75 size-exclusion column (GE Healthcare Bio-Sciences) pre-equilibrated with 50 mM sodium phosphate (pH 7.4). The protein was eluted

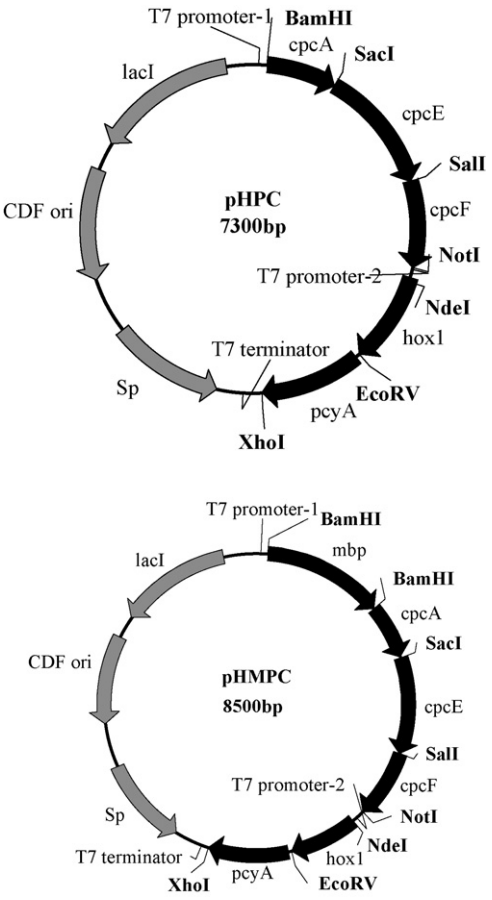

Fig. 1. Gene and restriction maps of expression plasmids pHPC and pHMPC.