List of Publications


- Parmar A., Singh N.K., Patel T., Tripathi A., Chruvattil R., Gupta S. and Madamwar D. Toxicity studies of phycoerythrins from three different from three different cyanobacterial cultures. *Manuscript under preparation*.


193
Optimization of medium components for increased production of C-phycocyanin from Phormidium ceylanicum and its purification by single step process

Iraj Kumar Singh, Asha Parmar, Datta Madamwar*

R&D School of Biosciences, Sardar Patel Maidan, Vadtal Road, Satellite Campus, Post Box No. 39, Sardar Patel University, Vallabh Vidyanagar 388120, Gujarat, India

**ABSTRACT**

Phycocyanin is a major protein produced by cyanobacteria, but very few phycocyanin-producing strains have been reported. In the present study, response surface methodology (RSM) involving a central composite design for four factors was successfully employed to optimize medium components for increased production of phycocyanin from *Phormidium ceylanicum*. The production of phycocyanin and interactions between sodium nitrate, calcium chloride, trace metal mix and citric acid stock were investigated and modeled. Under optimized condition *P. ceylanicum* was able to give 2.3-fold increase in phycocyanin production in comparison to commonly used BG 11 medium in 32 days. We have demonstrated the extraction, purification and characterization of C-phycocyanin using novel method based on filtration and single step chromatography. The protein was extracted by repeated freeze–thaw cycles and the crude extract was filtered and concentrated in stirred ultrafiltration cell (UFC). The UFC concentrate was then subjected to a single ion exchange chromatographic step. A purity ratio of 4.15 was achieved from a starting value of 1.05. The recovery efficiency of C-phycocyanin from crude extract was 63.50%. The purity was checked by electrophoresis and UV–Vis spectroscopy.

* Corresponding author. Tel.: +91 02692 229380; fax: +91 02692 231042/236475. E-mail addresses: nirajbiotech@gmail.com (N.K. Singh), parmar.asha@gmail.com (A. Parmar), datta.madamwar@yahoo.com (D. Madamwar).

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**INTRODUCTION**

Cyanobacteria are the oldest known oxygenic organisms, which y their photosynthetic activity probably made a fundamental contribution to the development of the present oxygen-enriched atmosphere (Zolla and Blanchetti, 2001). Cyanobacterial light harvesting pigments are chlorophyll-a, carotenoids and phycobiliproteins. Of these pigments, phycobiliproteins are a group of intensely colored proteins that occur only in cyanophyceae, rhodophyceae and cryptophyceae and can be subdivided into three main groups: phycocyanin (PC - dark cobalt blue - $\lambda_{max} = 610-620 \text{ nm}$), allophycocyanin (APC - brighter aqua blue - $\lambda_{max} = 650-655 \text{ nm}$) and phycoerythrin (PE - bright pink - $\lambda_{max} = 540-570 \text{ nm}$). They are organized in supramolecular aggreagates as phycobilisomes in order to maximize energy transfer to the chlorophyll-protein complexes located at the thylakoid membrane (Reis et al., 1998).

Because of the protein nature, unique color, fluorescence and antioxidant properties a wide range of applications of phycobiliproteins are possible especially in diagnostics, biomedical research and its purification by single step process (Mohana et al., 2003). The main application of phycobiliproteins is as fluorescent markers of cells and macromolecules in biomedical research (Bermijo et al., 2003). Recent studies have shown their applicability in immunomodulating and anticarcinogenic activities as well as natural dyes in food and cosmetics, replacing the synthetic colourants (Roman et al., 2002).

Cyanobacterial phycocyanin (C-PC) is the major phycobiliprotein in *P. ceylanicum*. This blue colored red fluorescing biliprotein was first reported in 1928 by Lemberg (Patil et al., 2006). It comprises of a protein component and a chromophore, and the protein moiety consists of $\alpha$ and $\beta$ subunits each having the molecular weight in the range of 15–20 kDa (MacColl, 1998).

Very few strains of cyanobacteria have the ability to produce high quantity of phycocyanin. There exists a need to look for high yielding strain and improve its production by manipulation of cultural conditions. The statistical experimental design can help to increase the phycocyanin yield at low cost. The conventional and classical method for optimizing medium components for protein production by 'one variable at a time' approach involves varying a single independent variable, while maintaining the others at a constant level. This one-dimensional approach is laborious and time consuming especially for large number of variables, and does not consider potential interactions among them. A statistical experimental design like the response surface methodology (RSM) overcomes the limitations of the classical method (Mohana et al., 2003).
NOTE

ALLOPHYCOEYANIN FROM A LOCAL ISOLATE GEITLERNEMA SP. A28DM (CYANOBACTERIA): A SIMPLE AND EFFICIENT PURIFICATION PROCESS

Asha Parmar, Niraj Kumar Singh, and Datta Madamwar

BRD School of Biosciences, Sardar Patel Maidan, Vadatal Road, Satellite Campus, Post Box No. 39, Sardar Patel University, Vallabh Vidyanagar 388120, Gujarat, India

Allophycocyanin (APC) is the least-studied cyanobacterial bile-pigment invariably present within the phycobilisome core of cyanobacteria. In the present study, we describe a simple, cost-effective, and reproducible method for the purification of APC from a local isolate, *Geitlerinema* sp. A28DM. The pigment was extracted from the algal biomass and precipitated with 0.25% aqueous solution of the highly aromatic cationic dye "ethodin." The precipitated APC was then subjected to a single size-exclusion chromatographic step using Sephadex G-100. Pure cyanobacterial APC (C-APC) ($A_{652}/A_{280}$ of 3.2) was obtained and characterized by its absorption spectrum with maximum at 652 nm and a shoulder at 620 nm, and by SDS-PAGE, showing two bands with molecular masses of 15 and 17.5 kDa, corresponding to $\alpha$ and $\beta$ subunits of the biliprotein. The final yield of C-APC was 66% from its content in the crude extract. The procedure appears to be promising for wider applications and larger production of APC.

Key index words: allophycocyanin; ethodin; *Geitlerinema*; gel permeation chromatography; purification

Abbreviations: APC, allophycocyanin; ASN, artificial salt nutrient; C-APC, cyanobacterial allophycocyanin; C-PC, cyanobacterial phycocyanin; IEF, isoelectric focusing; IPG, immobilized pH gradient; PC, phycocyanin; PE, phycoerythrin

Phycobiliproteins are homologous chromoproteins that constitute the phycobilisomes, the light-harvesting complexes of the photosynthetic apparatus in the red (Rhodophyta), cyanobacterial (Cyanophyta), and cryptomonad (Cryptophyta) algae (Bogorad 1975, Brown et al. 1975, Simo et al. 2005). Purified phycobiliproteins have a wide range of applications, such as colorants in food and cosmetics, and as labels in different fluorescence techniques (Roman et al. 2002). Phycobiliproteins also have important medical and pharmacological properties (Romay et al. 1998). Recent studies have shown that they also have hepatoprotective (Vadiraja et al. 1998), anti-inflammatory (Romay et al. 1998, González et al. 1999, Reddy et al. 2000), and antioxidant properties (Bhat and Madyastha 2000, Zhang et al. 2000, Soni et al. 2008).

Phycobiliproteins are assignable to three special subclasses: allophycocyanin (APC; $\lambda_{\text{max}}$: 650-655 nm), phycocyanin (PC; $\lambda_{\text{max}}$: 610-620 nm), and phycoerythrin (PE; $\lambda_{\text{max}}$: 540-570 nm), of which APC is invariably present within the core (Gantt et al. 1976, 1977, Koller et al. 1978, Bryant et al. 1979). APC is the most efficient biliprotein for energy transfer (Lemasson et al. 1973, Gantt et al. 1976, Koller et al. 1978, Bryant et al. 1979) and is the key pigment in funneling excitation energy from the other biliproteins into the chl of the PSII. Despite its obvious importance, APC still remains the least-studied phycobiliprotein.

Various methods have been reported for the purification of C-APC, but all of these involve a large number of chromatographic steps (Troxler et al. 1980, Zilinskas 1982, Gombos et al. 1983) usually in combination with ammonium sulfate precipitation (Brown et al. 1975, Gysi and Zuber 1976). Apart from being time consuming, ammonium sulfate precipitation may hinder the future crystallization process because of the difficulty of complete removal of salt by dialysis or even by desalting column (Singh et al. 2009). As general information, even a single increase in chromatography step can reduce ~20% of the protein of interest (Soni et al. 2008). We, therefore, looked to develop a method that would be simple, efficient, and easy to perform without compromising the purity and yield. Here, we used ethodin (6,9-diamino-2-ethoxyacridine lactate monohydrate) as an attractive alternative for the precipitation of APC. Ethodin is a strongly yellow-colored, highly aromatic cationic dye that complexes with negatively charged...
Structure of the novel 14 kDa fragment of α-subunit of phycoerythrin from the starving cyanobacterium *Phormidium tenue*

Badrish R. Soni a, Md. Imtaiyaz Hasan b, Asha Parmar a, Abdul S. Ethayathulla a, Ramasamy P. Kumar b, Niraj K. Singh a, Mau Sinha b, Punit Kaur b, Savita Yadav b, Sujata Sharma b, Datta Madamwar a, Tej P. Singh b,*

a BRD School of Biosciences, Sardar Patel University, Vallabh Vidyanagar 388120, India
b Department of Biophysics, All India Institute of Medical Sciences, New Delhi 110029, India

**Abstract**

The rod-like phycobilisome (PBS) in cyanobacterium is the light-harvesting complex of phycoerythrin (PE), phycoerythrin (PC) and allophycocyanin (APC). The orderly degradation of PBS was observed under starvation conditions. A 14 kDa truncated fragment of α-subunit of PE (F-αPE) was identified from the degraded product. F-αPE was purified to homogeneity, sequenced and crystallized. The merohedrally twinned crystals with a twinning factor of approximately 0.5 were obtained. The crystal structure of F-αPE was determined with molecular replacement method using detwinned data and refined to an Rfactor of 23.2% (Rfree = 27.6%). The structure consisted of two crystallographically independent molecules in the asymmetric unit. The two molecules were designated as molecules A and B with a buried area of 200Å² at the interface. The structure of F-αPE consists of seven α-helices A, B, E, F, P, G and H. The first 31 N-terminal residues that fold into parallel α-helices X and Y in other PEs are not present in the amino acid sequence of F-αPE. Both molecules, A and B contain two chromophore ligands, PEB1 and PEB2 in each. These are covalently linked to the polypeptide chain through Cys82 and Cys139, respectively. The superimposition of Cα tracings of molecules A and B shows an r.m.s. shift of 1.0 Å indicating that the structures of two independent molecules are very similar. The degradation of phycobilisome proteins under starvation stress seems to occur to supplement the requirement of amino acids for protein synthesis and to reduce the absorption of light energy.

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1. Introduction

The light-harvesting system in cyanobacterium is formed by the rod-like phycobilisomes (PBS). This blue-colored multiprotein assembly is arranged in a certain order where phycoerythrin (PE) is located at the tip of the rod, phycoerythrin (PC) is situated in the middle and allophycocyanin (APC) is placed at the core which is attached to the thylakoid membrane (Glazer, 1982). The purpose of this assembly is to increase the efficiency of photosynthetic organism growing in low light regions (MacColl, 1998). Both APC and PC have covalently bound phycocyanobilin (PCB) prosthetic groups while PE has covalently attached phycoerythrobilin (PEB) groups whereas in the case of red algal phyocyanin (R-PC), found in certain red algae, both PCB and PEB chromophores are found (Wedemayer et al., 1992). The energy transfer in the organism proceeds successively in the direction from PE → PC → APC → chlorophyll a, with an overall efficiency of almost 100% (Gantt and Lipschultz, 1973). All the known phycobiliproteins are composed of two different α- and β-subunits. These are further arranged in the form of dimers of trimers (αβ)3 forming a hexamer, (αβ)6. These trimers and hexamers form discs and assemble themselves into rod-like organization (Glazer and Hixson, 1977). Although these essential multiprotein assemblies form a stable arrangement, they undergo degradation when the organisms are starved for nutrition (Lichtlé et al., 1996). The process of degradation can be observed visually due to a change in the color from blue-green to yellow-green. However, the molecular basis of degradation and the role of surviving polypeptides is unclear. Presumably, degradation occurs to reduce the absorption of excess light energy under the stress conditions and also to provide amino acids to sustain the limited protein synthesis. In order to characterize the surviving polypeptides for gaining insights into the nature of molecular assembly and the mode of association and possibly the role of the polypeptide fragment of αPE during the state of starvation, the crystal structure of F-αPE has been determined.
Purification, characterization and comparison of phycoerythrins from three different marine cyanobacterial cultures

Asha Parmar, Niraj Kumar Singh, Avani Kaushal, Sagar Sonawala, Datta Madamwar*

BRD School of Biosciences, Sardar Patel Maidan, Vadetal Road, Satellite Campus, Post Box No. 39, Sardar Patel University, Vallabh Vidyanagar 388 120, Anand, Gujarat, India

A B S T R A C T

The present study is focused on purification, characterization and comparison of phycoerythrins from three different marine cyanobacterial cultures – Phormidium sp. A27DM, Lyngbya sp. A09DM and Halomicronema sp. A32DM. ‘Phycoerythrin’ was successfully purified and characterized. On SDS–PAGE, the PE purified from all three young cultures showed four bands – corresponding to α and β subunits of each of PE-I and PE-II. However, phycoerythrin purified after prolonged growth of Phormidium sp. A27DM and Halomicronema sp. A32DM showed only one band corresponding to 14 kDa whereas Lyngbya sp. A09DM continued to produce uncleaved phycoerythrin. The absorption spectra of purified PEs from all the three young and old cultures showed variations however the fluorescence studies of the purified PEs in all cases gave the emission spectra at around 580 nm. The described work is of great importance to understand the role of phycoerythrin in adapting cyanobacteria to stress conditions.

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1. Introduction

Cyanobacteria, prominent constituents of marine biosphere, account for a significant percentage of oceanic primary productivity, and are among the oldest life forms (about 3.5 billion years ago) on earth capable of doing oxygenic photosynthesis, similar to the process found in higher plants (Guan et al., 2007). Phycoerythrin, the oldest and major light-harvesting antennae, are multimolecular highly organized complexes of various phycoerythrin proteins and linker polypeptides. They are highly diverse in their structure and pigment composition in cyanobacteria, red algae and cryptomonad (Grossman et al., 1995; Guan et al., 2007). The major phycoerythrin proteins are phycoerythrin (PE, λmax: 540-570 nm), phycocyanin (PC, λmax: 610-620 nm) and allophycocyanin (APC, λmax: 650-655 nm) based on the spectral properties. The basic building block of these phycoerythrins is made up of dissimilar polypeptide chains (αβ) (Klotz et al., 1986), belonging to two families (α and β) probably originating from a common ancestor but apparently diverged early in evolution (Apt et al., 1995; Thomas and Pasaquet, 1999). These chains are generally present in equimolar amounts (Bernard et al., 1992) and are generally organized as trimeric (αβ)3, or as hexameric (αβ)6 discs. These discs are stabilized by linker polypeptides which ensure the correct structural assembly of phycoerythrin components and hold the complex together (Glazer et al., 1985; Glazer, 1989; Wilbanks and Glazer, 1993; MacColl, 1998; Six et al., 2005). Four isomeric tetrapyrroles (bilins), linked to the polypeptides through thioester linkages, function as the light-harvesting chromophores of phycoerythrins (Ong and Glazer, 1991). The nature and arrangement of the bilins in the individual phycoerythrins as well as the arrangement of the various phycoerythrins in the phycoerythrin is such as to ensure directional transfer of excitation energy from any point within this macromolecular complex towards the reaction center. The spectroscopic property of these tetrapyrroles are primarily a function of their covalent structure but are strongly influenced by their conformation and environment within the native phycoerythrins (Glazer, 1989; Ong and Glazer, 1991).

The intensely red phycoerythrins serve as major light-harvesting proteins in numerous cyanobacteria (Schoenlieber et al., 1994). Phycoerythrin extends the light-harvesting capability of the phycoerythrin into the green region of the visible light spectrum, and its location at the phycoerythrin periphery reflects the position of phycoerythrin in the sequential energy transfer pathway, PE–PC–APC–Chl α (Anderson et al., 1990).

High phycoerythrin content is a distinguishing adaptive characteristic of marine unicellular cyanobacteria (Waterbury et al., 1979; Wilbanks et al., 1991). In their natural habitat, these strict photoautotrophs are exposed primarily to blue-green light with maximum intensity near 500 nm which is strongly absorbed by phycoerythrins (Waterbury et al., 1986). The marine strains contain two different kinds of phycoerythrins, whereas all other cyanobacteria

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* Corresponding author. Tel.: +91 02692 229380; fax: +91 02692 231042/236475.
E-mail addresses: parmar.asha@gmail.com (A. Parmar), nirajbiotech@gmail.com (N.K. Singh), datta_madamwar@yahoo.com (D. Madamwar).

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In this study, we describe a series of experiments presenting the biochemical evidence for the cleavage of an intact phycoerythrin (α and β subunits) to phycoerythrin with only fragmented α-subunit, which is also functional, in Phormidium sp. A27DM. Culture, whether grown in different conditions (static or sparging) or in medium with different pH, produced truncated phycoerythrin. This indicated that the growth conditions or pH of medium did not lead to cleavage of phycoerythrin. Culture when grown in the medium lacking NaN03 started producing truncated phycoerythrin after 9 days of growth only, thus proving that nitrogen depletion was playing a role in phycoerythrin cleavage. In vivo and in vitro experiments with different proteases also resulted in production of truncated phycoerythrin. And on the addition of different protease inhibitors intact phycoerythrin formation was taking place even after 60 days of growth, thus indicating the possible role of proteases in phycoerythrin cleavage. Moreover, purified intact PE when stored at 4°C also gets cleaved to 14kDa fragment. However, when glacial acetic acid was added to phycoerythrin no cleavage was observed even after 4 months of storage. The events indicated that production of truncated PE is meant for continuing the absorption of light under stress conditions.

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