CHAPTER 3

Purification and characterization of allophycocyanin from Geitlerinema sp. A28DM

Part of this chapter has been published as:

3.1 Introduction

Phycobiliproteins are assignable to three special subclasses: allophycocyanin (APC; \( \lambda_{\text{max}}: 650-655 \text{ nm} \)), phycoerythrin (PE; \( \lambda_{\text{max}}: 540-570 \text{ nm} \)) of which APC is invariably present within the core (Gantt et al., 1976; Gantt et al., 1977; Koller et al., 1978; Bryant et al., 1979). Allophycocyanin is the most efficient biliprotein for energy transfer (Lemasson et al., 1973; Gantt et al., 1976; Gysi et al., 1978) and is the key pigment in funneling excitation energy from the other biliproteins into the chlorophyll of the photosystem II. Despite its obvious importance, APC still remains the least studied phycobiliprotein. This may be largely because of its miniscule quantity as APC accounts on a weight basis for 10% or less of the total cellular phycobiliproteins. Consequently, its absorption maxima in whole cells or crude extract is largely masked by much greater absorbancy of PC and of chlorophyll holochromes in this region (Gysi et al., 1978).

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 sulphate precipitation (Gysi and Zuber, 1976). Apart from being time consuming ammonium sulphate precipitation may hinder the future crystallization process because of the difficulty in 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 about 20% of protein of interest (Soni et al., 2006). Therefore a method was developed, which would be simple, efficient and easy to perform without compromising the purity and yield, wherein ethodin (6, 9-diamino-2-ethoxyacridine lactate monohydrate) was used as an attractive alternative for the precipitation of APC. Ethodin is strongly yellow coloured, highly aromatic cationic dye that complexes with negatively charged proteins. Ethodin has shown to have certain pharmacological applications, therefore very unlikely to be toxic (Rudolph et al., 1997). There are several reports showing the capability of ethodin as a precipitant for the protein purification (Miller, 1959; Berns, 1967; Neurath and Brunner, 1969; Minkova et al., 2003; Persson and Lester, 2004; Minkova et al., 2006).
permeation chromatography has been widely successful in protein purification and has the advantage of not being dependent on the net charge of the protein concerned.

The present study describes for the first time the purification of allophycocyanin from *Geitlerinema* sp. A28DM by a simple, inexpensive and efficient procedure consisting of size exclusion chromatographic step after treatment with ethodin.

### 3.2 Materials and methods

#### 3.2.1 Chemicals

Sephadex G-100 powdered matrix (bead diameter 20-300 μm and fractionation range 4-150 kDa) was purchased from GE Healthcare UK Limited (Little Chalfont, Buckinghamshire, UK), ethodin from Fluka Chemei GmbH (Industriestrasse 25, Buchs SG, Switzerland), protein molecular mass standard from Bangalore Genei (Bangalore, Karnataka, India), bis-acrylamide from Himedia (Mumbai, Maharashtra, India), sodium dodecyl sulphate and electrophoresis grade acrylamide from Merck (Darmstadt, Hesse, Germany). IPG strip from BioRad (Hercules, California, USA). All other chemicals were ultra pure or molecular biology grade and were used without further purification.

#### 3.2.2 Organism

##### 3.2.2.1 Isolation and growth conditions

The isolation and purification of the culture were carried out according to Shah et al. (2001). Twenty five cyanobacterial cultures were isolated from the sandy shores parallel to the coast indented by the estuarine mouth of river Tapi, Gujarat, India. They were cultivated in ASN III medium (Waterbury and Stanier, 1981) with 12-h light/12-h dark cycles at 27°C ± 2°C; illumination of 36 W white fluorescent lamps at a flux density of 130 μmol photons·m⁻²·s⁻¹ measured at the surface of the flask. Screening of the cultures was done and the cyanobacterium having maximum C-APC producing ability was selected.

##### 3.2.2.2 Identification

Genomic DNA was extracted from culture A28DM as described in Wu et al. (2000) and its 16S rRNA gene was amplified in a 30 μl PCR reaction consisting of 1X buffer.
(10 mM Tris pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100), 0.33 mM each of dNTPs, 0.66 μmoles each of custom synthesized universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), and 1.5U of Taq DNA polymerase. Amplification program was performed with initial denaturation step at 94°C for 3 min; followed by 30 cycles of 1 min denaturation step at 94°C, 1 min annealing step at 55°C and 1.2 min elongation step at 72°C followed by a final extension step at 72°C for 20 min using Biorad iCycler version 4.006 (Biorad, USA). The purified ~1.5 kb PCR product was sequenced by automated DNA Analyser 3730 using ABI PRISM® BigDye™ Terminator Cycle Sequencing 3.1 (Applied Biosystems, Foster City, CA). Full length 16S rRNA gene sequence was analyzed using SEQMATCH program at Ribosomal Database Project (RDP-II) to identify the organism.

3.2.3 Extraction and Purification of APC

One gram of twenty day grown wet algal biomass was washed with 10 mM potassium phosphate buffer (pH 6.7) containing 150 mM NaCl and 3 mM sodium azide. It was resuspended in 55 ml of the phosphate buffer. The suspension was frozen at -30°C overnight, thawed at 4°C for 2 hrs and incubated at 32°C for 90 min under continuous shaking conditions, left at 4°C overnight and then centrifuged (Kubota 6500, Bunkyo-Ku, Tokyo, Japan) at 17,000 × g for 40 min. The supernatant was termed as crude extract. All proceeding centrifugations were carried out at 10,000 × g for 40 min.

3.2.3.1 Treatment with ethodin

Aqueous ethodin solution (0.25%) was added to the crude extract in a ratio of 1:10. The resultant solution after mixing was left at 4°C overnight and then centrifuged. The pellet was resuspended in the phosphate buffer and allowed to stand overnight at 4°C without disturbance followed by centrifugation. The pellet was discarded and the supernatant termed as ethodin treated extract was subjected to size exclusion chromatography column.

3.2.3.2 Size exclusion chromatography

Ethodin treated extract after filtration (Whatman filter paper 1) was applied on a Sephadex G-100 column (150 cm x 1.5 cm, bed height 105 cm) pre-equilibrated and
eluted with the phosphate buffer. The flow rate was maintained at 60 ml h\(^{-1}\) using peristaltic pump (Model P1, Pharmacia, Sweden). Aqua blue coloured eluate was collected as 1 ml fractions.

3.2.4 Experimental estimations and analyses

The cell dry weight was determined according to Boussiba and Richmond (1980). The protein contents were determined by the method of Lowry et al. (1951), with BSA as the standard. Phycobiliprotein content was estimated using equations of Seigelman and Kycia (1978). The absorption overlay spectra of C-APC from *Geitlerinema* sp. A28DM at each step of purification was recorded on UV-Visible spectrophotometer (Analytik Jena AG Specord\textsuperscript{®} 210, Germany).

3.2.4.1 Electrophoretic analyses

*Non-denaturing gel electrophoresis*

Native polyacrylamide gel electrophoresis was carried out in a vertical slab gel apparatus (Mini Protean III, Biorad, Hercules, California, USA). The gel (1.0 mm thick) consisted of 5\% stacking and 10\% resolving polyacrylamide [25 mM Tris-Cl (pH 8.3) and 250 mM glycine]. Samples were prepared in 10 mM Tris-Cl (pH 6.8), 2\% (v/v) glycerol and 0.009\% (w/v) bromophenol blue. The separation was carried out at 4\°C, at 80 V for the initial 30 min and then at 100 V till the end of the run. The bands were visualized by silver staining (Garfin, 1990).

*Denaturing gel electrophoresis*

Denaturing gel electrophoresis was also carried out with a vertical slab gel apparatus (Mini Protean III, Biorad, Hercules, California, USA) with a stacking gel 5\% and resolving gel of 15\% acrylamide concentration. The gel prepared was 1.0 mm thick, containing 0.1\% (w/v) SDS. The samples were incubated with 2\% (w/v) SDS, 10\% (v/v) glycerol, 4.5\% (v/v) β-mercaptoethanol, 0.025\% (w/v) bromophenol blue and 60 mM Tris-Cl (pH 6.8) for 10 min in a boiling water bath. The gels were run at room temperature using a Laemmli buffer system (Laemmli, 1970) at 80 V for initial 30 min and then at 100 V till the end of the run. The bands were visualized by silver staining. PMWL 105978 (Genei, Bangalore, Karnataka, India) was used as the molecular weight marker [composition- ovalbumin (M\(_r\) 43 kDa), carbonic anhydrase
Chapter 3: Allophycocyanin from a., (Mr 29 kDa), soyabean trypsin inhibitor (Mr 20.1 kDa), lysozyme (Mr 14.3 kDa), aprotinin (Mr 6 kDa) and insulin (Mr 3.5 kDa)]. 10 µg protein was loaded in each well.

**Zinc acetate staining**
The resolved 10% native and 15% SDS gels were soaked in 20 mM zinc acetate solution for 5 min at room temperature (Brekelman and Lagarias, 1986). The bilin fluorescence was observed under UV light using AlphaEase FC Imaging System (Alpha Innotech, San Leandro, California, USA).

### 3.2.4.2 Isoelectric focussing

The isoelectric point of the purified C-APC was determined using precasted 3-10 pH linear gradient, 17 cm IPG readystrip (Biorad, USA), on protean IEF cell (Biorad, USA) following the manufacturer’s instructions. The temperature was set at 20°C during the entire run. Active rehydration of IPG strip was done at 50 V for 12 h. Focussing was done at 250 V for the first 15 min, 10,000 V for next 3 h and final focussing was done till the end of total 40,000 Vh. Band of C-APC was visualised by inherent aqua blue colour.

### 3.3 Results and discussion

#### 3.3.1 Identification

The 16S rRNA gene of the selected strain was sequenced (1501 bp) and identified by using SEQMATCH program at Ribosomal Database Project (RDP-II). The nearest neighbour was found to be *Geitlerinema* sp. Flo1 with a score of 0.992 and hence in the present study the selected is referred to *Geitlerinema* sp. A28DM. The sequence has been submitted to Genbank and the accession number is FJ410907.

#### 3.3.2 Extraction and purification of APC

Generally described methods for the purification of allophycocyanin are combination of chromatographic steps along with ammonium sulphate precipitation. The major limitations of these methods are the non-scalability and the length of time required to complete the process. Hence ethodin was used as an alternative precipitating agent. The absorption spectrum of the crude extract of *Geitlerinema* sp. A28DM (Fig. 3.1) showed the presence of C-PC peak and a shoulder peak at 652 nm indicating the presence of C-APC corroborating with the results reported by MacColl (2004).
data of C-APC purification from *Geitlerinema* sp. A28DM are summarized in Table 3.1. The total protein content of the crude extract was 28.2 mg including that of C-APC (2.3 mg). The simplified procedure resulted in pure C-APC ($A_{652}/A_{280}$ ratio of 3.2) and the product content reached up to 96% from the total proteins. The final yield of C-APC was 66% which is significantly higher than the other reported values (Minkova et al., 2006). Phycobiliprotein content (C-APC) at each step is shown in Table 3.1.

**Fig. 3.1:** UV-Visible absorption overlay spectra of C-APC from *Geitlerinema* sp. A28DM at each step of purification. Crude extract (red); Ethodin treated extract (green); Gel permeation chromatography eluate (blue).

**Table 3.1** Data of purification of C-APC from *Geitlerinema* sp. A28DM.

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Volume (ml)</th>
<th>Total Protein Content (mg)</th>
<th>Total C-APC Content (mg)</th>
<th>C-APC from Total Protein (%)</th>
<th>Impurities (%)</th>
<th>$A_{652}/A_{280}$</th>
<th>Yield of C-APC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>55</td>
<td>28.16</td>
<td>2.31</td>
<td>8.2</td>
<td>91.8</td>
<td>0.22</td>
<td>100</td>
</tr>
<tr>
<td>Ethodin treated crude</td>
<td>20</td>
<td>4.02</td>
<td>1.94</td>
<td>48.2</td>
<td>51.8</td>
<td>0.83</td>
<td>84</td>
</tr>
<tr>
<td>Gel permeation chromatography</td>
<td>30</td>
<td>1.59</td>
<td>1.53</td>
<td>96.2</td>
<td>3.8</td>
<td><strong>3.2</strong></td>
<td>66</td>
</tr>
</tbody>
</table>
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The ethodin molecule is positively charged over wider pH range (Persson and Lester, 2004). However, changing the pH will change the charge on the proteins. Hence, it is likely that the pH of the buffer could have a major effect on the purification of C-APC upon precipitation with ethodin. The strategy for purification herein was based on the fact that there is co-relation between the pi of the protein and the pH range at which the protein precipitates. The pi of C-APC was found to be 4.7, which is in the range of other phycobiliproteins reported (Glazer, 1993), and the pH of the buffer used was 6.7. Since the protein becomes negatively charged at pH above pi, C-APC was precipitated after treatment with positively charged ethodin. As ethodin precipitates partly due to charged characteristics of the molecule, it seems probable that the conductivity of the sample might have an effect on the purity. Hence addition of low concentration of NaCl (150 mM) in the buffer considerably improved the effective interaction of ethodin (Persson and Lester, 2004) with the crude extract components, leading to a significant purification of C-APC.

The concentration of ethodin plays a major role in protein purification (Persson and Lester, 2004). This property was exploited in C-APC purification. Every slight addition of ethodin in the crude extract resulted in increased purification. This effect was noted till the concentration was increased up to 0.25%. However, further increase in ethodin concentration did not result in additional purification. Hence the concentration of ethodin used in the study was kept at 0.25%.

Most of the methods for separation and purification of proteins are based on surface features of proteins, or on net charge, or on bioproperties (affinity), or on the whole structure (shape & size). Since the methods exploiting surface features depend on solubility properties, such methods were not chosen for the separation, as both C-APC and C-PC pigment proteins are hydrophilic in nature. Methods based on net charge were eliminated as both the proteins are negatively charged. Exploitation of bioproperties can be a powerful method for separating the desired protein from others. But since a particular biological property of C-APC which differs from C-PC had not been studied, the property based on the whole structure was exploited. Thus C-APC was purified in just one step of gel permeation chromatography. It has an added
advantage of not damaging the function of the protein as there is no binding of proteins to the chromatographic support and hence, the loss of protein is minimal.

3.3.3 Characterization

The success of C-APC purification protocol was checked at each step by UV-Visible spectroscopy and gel electrophoresis. The purity of C-APC at each step of purification was recorded in term of purity ratio \( \frac{A_{652}}{A_{280}} \). The increase in the purity ratio at each step was verified by the overlay spectra (Fig. 3.1).

3.3.3.1 Non-denaturing gel electrophoresis

Non-denaturing gel electrophoresis of C-APC obtained after the last step of purification showed only one band (Fig. 3.2) suggesting the homogeneity of the purified protein.

\[ \text{Fig. 3.2: Silver stained 10\% Native gel electrophoresis of pure C-APC from Geitlerinema sp. A28DM. Presence of only one band indicated the homogeneity of C-APC.} \]

3.3.3.2 Denaturing gel electrophoresis

The purity of the isolated C-APC was also verified by SDS-PAGE where only its \( \alpha \) and \( \beta \) subunits (Fig. 3.3), having molecular masses of 15 and 17.5 kDa, respectively, are visible which are in agreement with the previously reported values estimated by SDS-PAGE (Troxler et al., 1980; Zilinskas, 1982; MacColl, 1998).
3.3.3.3 Zinc-acetate staining

Bilins of the proteins fluoresce orange in the presence of zinc ions under the UV radiation (Brekelman and Lagarias, 1986). The zinc-assisted fluorescence enhancement of the native-PAGE and SDS-PAGE confirmed that the native protein and both the subunits were bilin-linked. At the final stage of purification, only one fluorescence band was observed in the native-PAGE (Fig. 3.4) proving the integrity of the purified C-APC. To check that both the subunits are linked to bilins, the resolved 15% SDS-PAGE gel was soaked in 20 mM zinc acetate solution for 5 min at room temperature. The characteristic orange fluorescence was observed under UV light. Two fluorescence bands were seen in SDS-PAGE corresponding to its $\alpha$ and $\beta$ subunits (Fig. 3.5). There were no bilin-linked peptides present in standard molecular mass marker suggesting the specificity of bili-protein detection by this method.

![Fig. 3.3: Silver stained 15% SDS gel electrophoresis at each stage of purification of C-APC from *Geitlerinema* sp. A28DM. Lanes: (1) Protein molecular mass standard, (2) Crude extract, (3) Ethodin treated extract, (4) Pure allophycocyanin eluate from gel permeation chromatography. Pure allophycocyanin showed two bands of 15 and 17.5 kDa molecular masses corresponding to characteristic $\alpha$ and $\beta$ subunits, respectively.](image-url)
Fig. 3.4: Detection of biliprotein of *Geitlerinema* sp. A28DM by zinc-assisted fluorescence enhancement method as observed under UV light. 10% native-PAGE of purified C-APC showed only one band suggesting the homogeneity and integrity of subunits.

Fig. 3.5: Detection of biliproteins of *Geitlerinema* sp. A28DM by zinc-assisted fluorescence enhancement method at each step of purification as observed under UV light. SDS electrophoresis was run in 15% gel. Lanes: (1) Protein molecular mass marker, (2) Crude extract, (3) Ethodin treated extract, (4) Pure C-APC eluate from gel permeation chromatography.
3.4 Conclusion

In conclusion, this work presents a significant, cost effective and reproducible method for the purification of C-APC from Geitlerinema sp. A28DM with comparatively higher yield. Compared to other methods, our method offers the advantage of avoiding lengthy and laborious procedures like ammonium sulphate precipitation, dialysis and other chromatographic processes. Hence, the protocol could be suitable for wider applicability to other cyanobacteria and for larger scale production. Moreover, it allows to obtain pure pigment for a broad range of applications.
3.5 References


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