Purification and characterization of light harvesting proteins from *Phormidium rubidum*

Part of this chapter has been published as:

2.1 Introduction
The cyanobacterium *Phormidium rubidum* (hereafter *P. rubidum*), isolated from rocky coastline of Okha (Gujarat, India) (Shah et al., 2001) is chosen to study the structure of its phycobilisome (PBS) in present study for two reasons. First, it produces blue light absorbing the lately evolved component, phycoerythrin (PE) along with red-light absorbing phycocyanin (PC) and allophycocyanin (APC) (Parmar et al., 2011; Six et al., 2007). Second, *P. rubidum* belongs to marine type cyanobacteria, from which very less structural information of phycobiliproteins (PBP) (especially for PE and APC) is available in protein data bank (see Table 1.2).

Purification of homogeneous population of protein molecules is prerequisite to produce well-ordered diffractable quality crystals. Moreover, the ultra-pure PBP are required to test their antioxidant activity and associated therapeutic efficacy, the second aim of present thesis. Several methods have been reported for purification of PBP - PE (Mishra et al., 2011; Parmar et al., 2011), PC (Bermejo et al., 2006; Patil et al., 2006; Santiago-Santos et al., 2004; Zhang & Chen, 1999) and APC (Parmar et al., 2010). Major of them is mainly based on salt-induce precipitation and charge/size-based chromatography, focusing on the purification of single PBP at a time, whereas other PBP are discarded. Simultaneous purification of all three PBP is very difficult due to similarities in their molecular weights and surface charges. Some recently reported-protocols like aqueous two-phase separation (ATPS) demonstrates new way for separation and simultaneous purification of two PBP (Patil et al., 2008); however, it requires tedious downstream processing to remove precipitants from purified proteins. This may impair the oligomeric state of PBP that can decrease the crystallization efficiency and interfere in the antioxidant assay. Therefore, “the development of protocol for separation of all possible PBP” has been learned as very first step to start with.

We develop new precipitation techniques, in which, the ammonium sulphate-induced precipitation is taken as a primer and amended by addition of non-ionic detergent- Triton X-100 to alter the protein solubility dynamics. This new precipitation technique is further coupled with size and charge based chromatography to achieve highly pure PBP. The details and success of PBP purification through this technique and characterization of purified PBP is described in present chapter.

*This organism is also known by other synonyms, Lyngbya sp. A09DM, Lyngbya rubida A09DM, Phormidium rubidum A09DM*
2.2 Materials and methods

2.2.1 Growth condition

*P. rubidum* was cultivated in artificial salt nutrient (ASN) III medium (*Waterbury & Stanier*, 1981) with 12:12 h light: dark cycles at 27 ± 2°C under 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 flasks (*Soni et al.*, 2006; *Soni et al.*, 2008).

2.2.2 Extraction and purification of PBP

*Extraction*: Optimally grown cell mass was washed before re-suspending into the extraction buffer (20 mM potassium phosphate buffer, pH 7.2) for repetitive freezing-thawing to draw out intracellular proteins. Supernatant collected after removal of cell debris by centrifugation (17,000 × g, 4°C, 20 min) was labelled as ‘crude extract’.

*Fractionation*: Fractionation of PBP was done by four-step ammonium sulphate precipitation amended with Triton X-100 treatment as shown in Fig. 2.1. In step I, finely powdered ammonium sulphate was gradually added into the crude extract to obtain 20% saturation with continuous stirring for 1 h at 4°C. The red supernatant, obtained after centrifugation (17,000 × g, 4°C, 20 min) of step I mixture, was pooled and subjected to 40% ammonium sulphate saturation (step II). Red pellet and dark blue supernatant were obtained after centrifugation (17,000 × g, 4°C, 20 min). Red pellet was further processed for chromatographic purification to obtain pure PE. Triton X-100 (0.1%, w/v) was added to remaining dark blue supernatant and left overnight at room temperature in static condition (step III). Blue pellet and aqua blue supernatant were collected after centrifugation (17,000 × g, RT, 20 min). Blue pellet was further processed for chromatographic purification to obtain pure PC. Remaining aqua blue supernatant was saturated with 70% ammonium sulphate (step IV). Aqua blue pellet, obtained after centrifugation (17,000 × g, RT, 20 min), was further subjected for chromatographic techniques to get pure APC.

*Chromatographic purification*: Protein obtained after step II, III and IV were passed through gel permeation matrix, sephadex G-150 packed in the column (400 mm x 10 mm, bed height 320 mm). A 20 mM potassium phosphate buffer (pH 7.2) was used as a mobile phase with flow rate of 45 mL h⁻¹, maintained using peristaltic pump (Model P1, Pharmacia, Sweden). Elutes were collected in 1 mL fractions.
Addition of Triton X-100
Up to 0.1% final Concentration

40% Ammonium Sulfate fractionation

20% Ammonium Sulfate fractionation

70% Ammonium Sulfate fractionation

Figure 2.1. Schematic flow chart of fractionation of PE, PC and APC from *P. rubidum*

Elutes from sephadex G-150 were further purified by passing through diethylaminoethyl (DEAE)-cellulose (anion exchangers) column (80 mm x 25 mm, 60 mm bed height), pre-equilibrated with the mobile phase, 20 mM potassium phosphate buffer (pH 7.2). After washing with 10 bed volumes of the same buffer, the column was eluted with an elution buffer containing increasing step gradient of NaCl (from 0 to 0.5 M) in mobile phase with flow rate of 1 mL min⁻¹.
2.2.3 Assessment of purity, homogeneity and integrity of purified PBP

Spectroscopic analysis

The purified PE, PC and APC were analysed by recording the absorbance spectrum over 250-750 nm wavelength range on UV-visible spectrophotometer (Analytik Jena AG, Specord 210). The purity of PBP at each step of purification was recorded as ‘purity ratio’ calculated by formulas $A_{564}/A_{280}$ (for PE), $A_{616}/A_{280}$ (for PC) and $A_{653}/A_{280}$ (for APC) ($A_X$: Absorbance at X nm wavelength). The PE, PC and APC were exited at 559, 589 and 645 nm, respectively and assessed for successive emission band by fluorescence spectrophotometer (F-7000, Hitachi High Technologies) to verify the integrity of their fluorescence function.

Gel electrophoresis analysis

Purified PBP were characterized in terms of the molecular mass of their subunits and homogeneity by denaturing as well as non-denaturing polyacrylamide gel electrophoresis. All electrophoresis were carried out according to Singh et al. (2012) as and protein bands were visualized by silver staining according to Garfin (1990) and zinc-acetate staining according to Berkelman and Lagarias (1986). Standard marker proteins ranging from 6 kDa to 43 kDa (Aprotinin - 6 kDa, Lysozyme - 14.3 kDa, Soyabean Trypsin Inhibitor - 20.1 kDa, Carbonic Anhydrase - 29 kDa and Ovalbumin - 43 kDa) were used for molecular mass determination. Gel images were captured and analysed under AlphaEase FC imaging system (Alpha Innotech Corp., San Leandro, CA, USA).

2.2.4 Growth and protein measurements

Growth was measured in terms of total chlorophyll $a$ contents of cell mass. Chlorophyll $a$ was extracted from the cell mass according to method of De Marsac and Houmard (1988) and was estimated by absorbance at 665 nm on spectrophotometer (Spectronic 20D+, Spectronic Instruments Inc., New York, USA). Total protein contents were determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard. PE, PC and APC contents were calculated from UV – visible spectrum using Bennett and Bogorad (1973).
2.3 Results and discussion

2.3.1 PBP content at different stage of growth

The cell mass of *P. rubidum* after every 48 h of incubation was harvested and analysed for its growth and PBP content. The increase in chlorophyll *a* (growth) and PBP amount was found insignificant during first 4 days, which was followed by substantial increase in production of both (Fig. 2.2). Maximum production was recorded on 30th day of incubation and further incubation could increase neither chlorophyll *a* nor PBP content (Fig. 2.2). Hence, we took 30 days old culture for the extraction of PBP.

![Figure 2.2](image_url). Growth pattern in terms of chlorophyll (vertical bars) and concurrent PBP production profile (scattered line) of *P. rubidum*, grown for 40 days. A, growth period during which no increase in chlorophyll *a* and PBP content; B, rapid increase in chlorophyll *a* and concomitant increase in PBP content; C, no further increase in chlorophyll *a* as well as PBP content

2.3.2 Extraction, fractionation and purification of PBP

*Extraction:* Temperature for freezing and thawing were optimized and found best at -25°C and 4°C, respectively. Two such successive freeze-thawing cycles caused leaching out of an intracellular content from the cell. Brick red supernatant after removal of cell debris was collected and labelled as ‘crude extract’.

*Fractionation:* To separate PBP from one another and other impurities, the treatment with detergent-cum-hydrophobic chemical Triton X-100 was incorporated in traditional ammonium sulphate precipitation. The combined action of Triton X-100 and ammonium sulphate productively fractionated individual PBP in successive steps. Step I, initial 20% ammonium sulphate saturation precipitated bio-molecules other than
PBP along with suspended impurities. Step II, 20-40% ammonium sulphate saturation, precipitated only PE from the step I supernatant. Step III, an addition of Triton X-100 (0.1%) to step II supernatant, has precipitated PC. The final step IV, 70% saturation of step III supernatant, has precipitated APC and left other proteins in supernatant. Purity ratios established after fractionation were 1.59, 2.35 and 2.08 for PE (step II), PC (step III) and APC (step IV), respectively (Table 2.1).

Table 2.1. Summary of purification protocol in terms of protein content, purity and yield at each stage of purification

<table>
<thead>
<tr>
<th>Steps of Purification</th>
<th>Total protein (mg)</th>
<th>PE/PC/APC content (mg)</th>
<th>PE/PC/APC out of total protein (%)</th>
<th>Impurities (%)</th>
<th>Purity ratio $\frac{A_{max}}{A_{280}}$</th>
<th>Yield (%)</th>
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<tbody>
<tr>
<td>PE</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Crude</td>
<td>88.76</td>
<td>27.47</td>
<td>30.94</td>
<td>69.06</td>
<td>0.50</td>
<td>100.00</td>
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<tr>
<td>20-40 % ASC (step II)</td>
<td>35.24</td>
<td>23.76</td>
<td>67.42</td>
<td>32.58</td>
<td>1.59</td>
<td>86.49</td>
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<td>IEC eluates*</td>
<td>21.32</td>
<td>20.92</td>
<td>98.12</td>
<td>1.88</td>
<td>6.75</td>
<td>76.16</td>
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<tr>
<td>PC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude</td>
<td>88.76</td>
<td>20.43</td>
<td>23.02</td>
<td>76.98</td>
<td>0.21</td>
<td>100.00</td>
</tr>
<tr>
<td>20-40 % ASC + 0.1 % Triton X-100 (step III)</td>
<td>21.38</td>
<td>14.76</td>
<td>69.03</td>
<td>30.97</td>
<td>2.35</td>
<td>72.29</td>
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<tr>
<td>IEC eluates*</td>
<td>12.54</td>
<td>12.30</td>
<td>98.07</td>
<td>1.93</td>
<td>5.53</td>
<td>60.23</td>
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<tr>
<td>APC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude</td>
<td>88.76</td>
<td>6.55</td>
<td>7.38</td>
<td>92.62</td>
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<td>40-70 % ASC (step IV)</td>
<td>8.20</td>
<td>5.31</td>
<td>64.75</td>
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<td>2.08</td>
<td>81.20</td>
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<tr>
<td>IEC eluates*</td>
<td>4.84</td>
<td>4.71</td>
<td>97.31</td>
<td>2.69</td>
<td>5.43</td>
<td>71.91</td>
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</tbody>
</table>

*Ion exchange chromatography eluates

Presently used strategy of PBP purification is based on ‘surface hydrophobicity’ of protein and ‘hydrophobic resolution’ of solvent that determines ‘protein solubility’ in solution (Englard & Seifter, 1990). The alteration in ‘hydrophobic resolution’ makes some protein incompatible for solution and causes their exclusion as a precipitate or in second phase. Ammonium sulphate precipitation and aqueous two-phase separation (ATPS) (Patil et al., 2008), well established tools for PBP separation and purification (Soni et al., 2006) work on this principle. However, ammonium sulphate precipitation is not able to separate PE, PC and APC from one another because of its non-specificity to discriminate between their surface hydrophobic properties. On the other hand, ATPS can separate PE, PC and APC through phase separation, but requires tedious downstream processing for the removal of process components. The use of Triton X-100 along with ammonium sulphate precipitation provided better separation of PBP through the refinement of the microenvironment around protein molecules, which ultimately enhanced efficiency of solution to discriminate between PE, PC and APC.
In this mixture, ammonium sulphate decreasing water availability and Triton X-100 refining the hydrophobic resolution of saturated solution work in combinatorial manner to separate PE, PC and APC.

Chromatographic purification: PBP, once separated by above described fractionation, were passed through gel permeation resin sephadex G-150 and then further purified through diethyl amino ethyl cellulose (DEAE-cellulose) ion exchange resin. Pure PE, PC and APC having highest purity were collected separately and labelled as IEC elutes. Purity ratios established after chromatographic purification were 6.75, 5.53 and 5.43 for PE, PC and APC, respectively (Table 2.1). Furthermore, the purity was affirmed by manifestation of SDS-PAGE as only two band corresponding to α- and β-subunit of each PBP were observed (Fig. 2.3A) and by UV-visible spectrum as only single peak at relevant absorbance maximum of PBP dominated over the peak at 280 nm (Fig. 2.3B).

**Figure 2.3.** (A) Silver stained (S) and zinc acetate (Z) stained 15 % SDS - PAGE of protein molecular mass standard (Lane 1), crude extract (Lane 2), pure PE (Lane 3), pure PC (Lane 4) and pure APC (Lane 5) from *P. rubidum*. (B) UV-visible absorbance spectrum of crude extract, pure PE, pure PC and pure APC from *P. rubidum*.

### 2.3.3 PBP characterization and determination of purity

Purity was monitored by UV-visible spectroscopy at each step of purification. A sharp increase in a single peak at 564, 616 and 653 nm signified the prominent abundant of PE, PC and APC, while the decreases in absorbance peaks at 280 nm signified the removal of other cellular proteins (Fig. 2.3B). Native-PAGE provides the evidence of homogeneity and structure integrity of purified PBP by manifestation of single band in lane of each purified PBP (Fig. 2.4A). Molecular weight of α- and β-subunits of PE, PC
and APC, obtained from silver stained SDS-PAGE, were around 19.0 and 21.5 kDa (for PE), 17.5 and 19.0 kDa (for PC) and 15.5 and 17.0 kDa (for APC), respectively (Fig. 2.3A). Zinc acetate stained SDS-PAGE of purified PBP showed two distinct fluorescent bands under UV light, which confirmed the presence of chromophore(s) with each subunits of purified PBP (Fig. 2.3A). Purified PE, PC and APC showed successive emission band centred at 581, 645 and 658 nm when exited over the 559, 589 and 645 nm (Fig. 2.4B), respectively. These values revealing the integrity of fluorescence function of purified PBP are in correspondence with previous reports (Munier et al., 2014; Singh et al., 2012).

![Fluorescence emission spectrum of purified PE, PC and APC](image)

**Figure 2.4.** (A) Fluorescence emission spectrum of purified PE, PC and APC when exited at 559, 589 and 645 nm, respectively. Successive emission band in fluorescence spectrum indicated the integrity of fluorescence function of purified PBP. (B) Silver stained 12% Native-PAGE of purified PE, PC and APC. Single band in Native-PAGE of each PBP suggests their homogeneity and structural integrity.

With this protocol, purity ratios 0.50, 0.21 and 0.09 in crude, were improved up to 6.75, 5.53 and 5.43 for PE, PC and APC, respectively, which are comparable to the previously reported values (Bermejo et al., 2013; Liu et al., 2012; Ramos et al., 2011; Ramos et al., 2010; Song et al., 2013; Su et al., 2010). Moreover, this protocol proficiently recovered around 76.16% PE, 60.23% PC and 71.91% APC from crude extract. Present protocol provides an easiness of ammonium sulphate precipitation and efficiency of ATPS, which do not require an extensive downstream processing. The described method could be applicable to precipitate any protein from a mixture of proteins if proper concentrations of ammonium sulphate and Triton X-100 are taken in account with respect to solubility of proteins.
2.4 References


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