A stable and functional single peptide phycoerythrin (15.45 kDa) from *Lyngbya* sp. A09DM

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**Abstract**

A functional and stable truncated-phycoerythrin (T-PE) was found as a result of spontaneous in vitro truncation. Truncation was noticed to occur during storage of purified native-phycoerythrin (N-PE) isolated from *Lyngbya* sp. A09DM. SDS and native-PAGE analysis revealed the truncation of N-PE, containing \( \alpha \) (19.0 kDa) and \( \beta \) (21.5 kDa) subunits to the only single peptide of \( \sim 15.45 \) kDa (T-PE). The peptide mass fingerprinting (PMF) and MS/MS analysis indicated that T-PE is a part of \( \alpha \)-subunit of N-PE. UV-visible absorption peak of N-PE was found to split into two peaks (540 and 565 nm) after truncation, suggesting the alterations in its folded state. The emission spectra of both N-PE and T-PE show the emission band centered at 581 nm (upon excitation at 559 nm) suggested the maintenance of fluorescence even after significant truncation. Urea-induced denaturation and Gibbs-free energy (\( \Delta G^\circ \)) calculations suggested that the folding and structural stability of T-PE was almost similar to that of N-PE. Presented bunch of evidences revealed the truncation in N-PE without perturbing its folding, structural stability and functionality (fluorescence), and thereby suggested its applicability in fluorescence based biomedical techniques where smaller fluorescence molecules are more preferable.

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1. Introduction

Cyanobacteria are the most primitive among oxygenic photosynthetic bacteria and well known for their characteristic antenna system called phycobilisome (PBS) [1,2]. PBS is a giant light harvesting complex made up of phycobiliproteins (PBPs) and associated linker peptides. Morphologically, PBS contains two major sub-structures: core consists of 2/3/5 cylinders situated horizontally on the outer surface of thylakoid membrane and 6/8 rods radiating out from the core [3–5]. Rods always contain phycocyanin (PC, \( \lambda_{\text{max}} \sim 610–620 \) nm), and often also phycoerythrin (PE, \( \lambda_{\text{max}} \sim 540–570 \) nm); whereas core invariably contains allophycocyanin (APC, \( \lambda_{\text{max}} \sim 653 \) nm) [1]. The chromophores like phycoerythrobilin, phycocyanobilin, phycourobilin and phycoviolobilin attach covalently to the PBPs-apoliproteins and thereby direct the spectral properties of particular PBP [1,6]. Unique absorption and emission properties of PBP enable PBS to capture and funnel the sunlight energy in a unidirectional manner from PE \( \rightarrow \) PC \( \rightarrow \) APC \( \rightarrow \) chlorophyll a for photosynthesis [7]. Moreover, protein microenvironment, optimum geometry (distance and orientation) and the appropriate docking of adjacent sub-complexes within PBS govern the expression of overall spectral properties and energy transfer efficiency of PBS supra-molecule [1].

PE, the blue/green light absorbing PBP, is basically made up of two types of polypeptides called \( \alpha \) (\( \sim 164 \) amino acids) and \( \beta \)-subunit (\( \sim 171 \) amino acids) with 65–70% sequence identity among different species [8,9]. The \( \alpha \) and \( \beta \)-subunits, contain covalently attached chromophore/s, associate to construct \( \alpha \)-\( \beta \) dimer, the building block of PBP assembly [9]. *In vitro* proteolytic cleavage studies [10] and structural analysis [8] has shown the important role of N-terminal domain in the formation of stable \( \alpha \)-\( \beta \) dimer as well as in prevention of \( \alpha \)-\( \alpha \)/\( \beta \)-\( \beta \) homodimerization. The \( \alpha \)-\( \beta \) dimers form ring shaped trimmer with threefold symmetry. Trimmers congregate further in a face-to-face manner to form hexameric disk, serving as a structural unit in the peripheral part of PBS rod [3,11].

It is believed that PE cannot be functional without characteristic folding of chromophore/s appended \( \alpha \)- and \( \beta \)-subunits. Many reports [12–16] have described the purification and characterization of PE made up of two native folded subunits from both cyanobacteria and red algae. Moreover, available structural information also suggested the important role of molecular interactions between \( \alpha \)- and \( \beta \)-subunits of PE to associate productively within PBS [17–19]. Only few reports are available, which have
described the alternative forms of PE. Huber [20] described the PE, made up of three different peptides such as α-, β- and γ-subunits. On the contrary, Thomas and Passaquet [21] described PE, composed of only β-subunit from red algae. Steglich et al. [22] and Wiethaus et al. [23] have also described degenerated form of PE (made up of only β-subunit) as remnants of PBS upon high-light exposure. Furthermore, Soni et al. [8] and Parmar et al. [24] have reported fragmented-PE, composed of only truncated α-subunit. In the present study, we report one more variant of PE, obtained via truncation in native-PE (N-PE) (with α- and β-subunits) of *Lyngbya* sp. A09DM during in vitro storage condition. We have purified and characterized the folding, stability and functionality (fluorescence) of truncated-PE (T-PE) obtained as a part of N-PE.

2. Materials and methods

2.1. Culture, growth conditions and protein purification

*Lyngbya* sp. A09DM, a filamentous cyanobacterium was isolated from the rocky shores of Okha, Gujarat, India. It was grown in ASN III medium [25,26] and subjected for PE extraction and purification as described earlier [26,27]. Purified PE was concentrated and stored at 4 °C under dark condition, and labeled as native-PE (N-PE).

After fifty days of storage, N-PE truncated spontaneously to give single and smaller peptide. This single peptide was purified by passing the fifty days stored N-PE through gel permeation (Sephadex G-100) column. Fractions with high purity were collected, concentrated and labeled as truncated-PE (T-PE).

2.2. Characterization of T-PE and N-PE

2.2.1. Gel electrophoresis analysis

N-PE and T-PE were resolved on native- and SDS-PAGE as described earlier [26,28]. Proteins on resolved gels were visualized by silver and zinc-acetate staining as described earlier [29–31].

2.2.2. Protease assay

The presence of protease in PE – solution was traced by protease assay as described in Sarath et al. [32]. Casein was used as a substrate. Trypsin and Proteinase K were used as positive control.

2.2.3. Spectroscopic analysis

The absorbance spectra of purified T-PE and N-PE were recorded over 250–750 nm wavelength range using cuvette of 1 cm path length on UV–visible spectrophotometer (Specord 210, Analytik Jena AG, Jena, Germany). The purity of protein was probed as ‘purity ratio’, calculated by the formula $A_{560}/A_{280}$ ($A_{p}$ stands for absorbance at x nm wavelength) [26]. The fluorescence emissions of T-PE and N-PE (−0.5 mg/ml) were recorded to verify their functionality upon excitation at 559 nm by fluorescence spectrophotometer (F-7000, Hitachi High Technologies Corp.).

2.2.4. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis (MALDI-TOF-MS)

MALDI-TOF of T-PE was performed as described by Gupta et al. [33] with slight modification. In brief, the purified T-PE was diluted to 10–15 pmol µl$^{-1}$ in potassium phosphate buffer (20 mM, pH 7.0) and 2 µl of diluted T-PE was mixed with sinapinic acid matrix (prepared in trifluoroacetic acid). This mixture was spotted on a target MALDI plate, allowed to dry at room temperature and analyzed by the MALDLYNX program.

2.3. Peptide mass fingerprinting (PMF)

2.3.1. Gel elution and trypsin digestion of T-PE and N-PE subunits

Purified T-PE and N-PE (50 µg of each) were loaded and resolved on 15% PAGE under denaturing condition. Proteins bands on the resolving gel were made visual by coomassie brilliant blue G250 staining. Stained bands were cut and digested individually by Trypsin-Gold (Promega Corp., Madison, WI, USA) according to manufacturer’s protocol. Digested samples were purified by passing through Millipore® ZipTips (Sigma–Aldrich, USA) with TA buffer (made up of 1:1 ratio of 0.1% TFA and acetonitrile) [34].

2.3.2. Mass spectrometry

A matrix solution was prepared by mixing α-cyano-4-hydroxycinnamic acid (5 mg ml$^{-1}$) in TA buffer containing purified peptide mixture. Matrix solution (2 µl) was applied to the MALDI target plate and allowed to dry before MS analysis. PMF spectra of α-N-PE, β-N-PE and T-PE were obtained in the positive reflection mode by the MALDI-TOF/TOF mass spectrometer (ULTRAFLEX III, Bruker Daltonics, USA). Spectra were recorded over the range of 0–4000 Da with an accumulation of 1000 shots for each sample [35]. MS/MS spectra of selected peptides were generated at 1 keV acceleration voltage by gas/air-collision induced dissociation under 10–6 Torr pressure [34].

2.3.3. Bioinformatics analysis

PMF spectrums were analyzed by Flex analysis software to extract peak list. Mascot (Matrix Science, UK) was used to find matches against the UniProtKB database. Mascot analysis was done with trypsin as the enzyme, carbamidomethylation (C) as a fixed modification, oxidation (M) as variable modifications, maximum missed cleavages as 1, peptide mass tolerance (MS) of ±100 ppm and fragment mass tolerance (MS/MS) of ±1 Da.

2.4. Chemical denaturation and renaturation study

2.4.1. Sample preparation

Urea was used to study chemical-induced denaturation of T-PE and N-PE. A solution of urea was prepared freshly in 20 mM potassium phosphate buffer (pH 7.2) as described in Pace [36]. The proteins were extensively dialyzed against the potassium phosphate buffer (20 mM, pH 7.2) to remove residual salts. Dialyzed proteins were quantified spectrophotometrically by using the molar absorption coefficient value (265,000 M$^{-1}$ cm$^{-1}$) for 565 nm [37]. Increasing amount of urea (0.2–9.0 M) was mixed with N-PE/T-PE and allowed to incubate for 45 min at 25 °C as described earlier [38]. Renaturation was also performed by following the same procedure, but the concentration of urea was decreased gradually by diluting the 9.0 M urea in the reaction mixture.

2.4.2. Measurement of UV–visible absorbance spectrum

Absorption spectra of denatured/renatured T-PE and N-PE were measured as described in Section 2.2.3. The protein concentration was used in the range of 0.2–0.4 mg ml$^{-1}$. All spectral measurements were done in triplicates.

2.4.3. Data analysis

The sigmoidal chemical-induced denaturation curve (change in molar extinction coefficient, $\Delta A$ vs. [urea], M) was plotted and analyzed for the deduction of $\Delta G^\circ$ (Gibbs free energy change for denaturation of protein), $m$ (slope of the plot of $\Delta G^\circ$, the Gibbs free energy change vs. [urea], i.e., $\Delta A$/$A$ vs [urea]) and $C_m$ (midpoint of denaturation curve, i.e., [urea] at which $\Delta G^\circ = 0$) [9]. The least-squares method was used to fit the data of the denaturation curve.
by assuming the linear relationship between $\Delta G_D$ and [urea], which was expressed by

$$y = y_N + y_0 \times \frac{\exp[-(\Delta G_D^0 - m[\text{Urea}])/RT]}{1 + \exp[-(\Delta G_D^0 - m[\text{Urea}])/RT]}$$  \hspace{1cm} (1)$$

where $y_N$ and $y_0$ are absorbance properties of the native (N) and denatured (D) protein molecules under identical experimental condition except [urea], $R$ is the gas constant and $T$ is the temperature in Kelvin (K).

3. Results and discussion

3.1. Truncation of native PE (N-PE)

We found spontaneous truncation in purified N-PE during long term in vitro storage at 4 °C. While monitoring the pattern of truncation, we noticed that N-PE containing $\alpha$ (19.0 kDa) and $\beta$ (21.5 kDa) subunit truncates to give a single peptide of ~15.5 kDa (T-PE) (Fig. 1A) after around 50 days of storage. However, no reduction in the size and intensity of T-PE band (~15.5 kDa) (Fig. 1B) upon further storage (of 200 days) revealed its stability over truncation phenomenon.

3.2. Purification and characterization of N-PE and T-PE

Freezing and thawing at ~25 °C and 4 °C, respectively, was found optimum to cause cell wall breakage and thus leaking out intracellular content. Brick red color supernatant after removal of cell debris was named as ‘crude extract’. Crude extract was further subjected to 20–70% ammonium sulfate fractionation, which resulted in the retention of all PE in the pellet upon centrifugation. The pellet containing PE was passed through gel permeation resin sephadex G-150 using potassium phosphate buffer (20 mM, pH 7.0) as the mobile phase. Elutes from gel permeation matrix was further purified by using diethyl amino ethyl-cellulose (DEAE-cellulose) matrix. PE was eluted by mobile phase containing 0.15 M NaCl in potassium phosphate buffer (20 mM, pH 7.0). Purified N-PE and T-PE did not show significant protease activity as well as color development during protease assay (Fig. 1C). These results significantly ruled out the possibility of presence of any protease in PE preparations. Furthermore, the purity assessment by SDS-PAGE (Fig. 1A) and UV–visible spectroscopy (Fig. 2 and Table 1) confirmed that N-PE and T-PE were very pure. The controlled truncation of N-PE in its pure form (without any external factor) suggested that the N-PE is might be truncated via some self-catalyzed and auto-governed mechanisms.

Zinc acetate stained SDS-PAGE showing the identical band pattern to that of silver stained gel revealed the presence of the chromophore/s with each subunit of N-PE and T-PE (Fig. 1A). However, the intensity of N-PE and T-PE band differed in zinc acetate stained native-PAGE under UV light illumination (Fig. 3). T-PE showed higher fluorescence intensity than that of the N-PE for

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**Fig. 1.** (A) Silver stained (S) and zinc acetate (Z) stained 15% SDS-PAGE of protein molecular mass standard (Marker), N-PE and T-PE. (B) Silver stained (S) and zinc acetate (Z) stained 15% SDS-PAGE of T-PE at every 25 days of interval. (C) Protease content in terms of unit activity and color development (inset) in control (A), trypsin (B), proteinase K (C), N-PE (D) and T-PE (E). N-PE and T-PE showing non-significant protease activity as well as color development indicated the complete absence of protease in PE preparations.

**Fig. 2.** UV–visible and fluorescence emission spectrum of N-PE (A) and T-PE (B) (excitation was provided at 559 nm in emission spectrum measurement).
identical protein load (Fig. 3). Variation in band intensity in native-PAGE is justified as the manifestation of altered chromophore/s accessibility and reactivity (related with its conformation) to zinc acetate in the altered folding of T-PE than that of N-PE. Truncation might alter the native folding of T-PE in a manner that either exposes chromophore/s on the outer solvent accessible surface or changes the chromophore/s conformation toward more reactive isoform.

The absorbance peak of N-PE was found to be split into two peaks (540 nm and 565 nm) upon truncation, which is similar to that of recombinant α-N-PE [39]. Appearance of slight fall at ∼550 nm in absorption of T-PE reflected the occurrence of conformational...
changes in its native folding to fulfill the absenteeism of lost amino acid residues after truncation (Fig. 2). However, T-PE showed the successive emission band centered at 581 nm when excited at 559 nm (Fig. 2B) similar to that of N-PE (Fig. 2A) and only α-N-PE [40]. Pattern of alteration occurring in absorption and fluorescence emission properties of PE upon truncation revealed the maintenance of its functionality even in perturbed folding.

MALDI-TOF spectrum of T-PE shows a significant peak corresponds to 15.45 kDa (Fig. 4) suggested the presence of single substantial forms of T-PE in solution. Another small peak corresponds to 14.87 kDa in MALDI-TOF is might be of chromophore/s detached PE-apoproteins, generated due to laser desorption.

3.3. T-PE is a part of α-subunit of N-PE

PMF of N-PE subunits and T-PE was performed to identify the origin of truncated subunit. PMF spectrum of truncated subunit showed high similarities with that of α-subunit as compared to
β-subunit of N-PE (Fig. 5). Amino acid sequences of two major peptides (1777 and 2431 Da) of trypsin digested T-PE were deduced from their MS/MS spectrums as shown in Fig. 6A and B. Moreover, Mascot similarity search has revealed 100% sequence similarities of these peptides with PE-α-subunit (accession no. P05098) in the UniProtKB database (Fig. 6C). Furthermore, we found significant loss of some peaks (1402, 1913, 2252, 3121, 3381 m/z) in the PMF spectra of T-PE while comparing it with that of α-N-PE. The peaks at ~2252 and ~3381 m/z correspond to 18thArg–37thArg and 14Met–33thArg oligopeptides, respectively, possibly produced from N-terminus of α-N-PE as a result of trypsin digestion. The results of PMF and MALDI-TOF analysis collectively suggested that the T-PE is produced from α-subunit of N-PE via truncation of 25–30 amino acids from N-terminus. Since α-subunit of PE has been reported to contain two chromophores attached with two conserved cysteine situated on 84/82 and 140/139 position [18,19,41], T-PE is assumed to still contain both chromophores according to the site of cleavage. However, this assumption has been already confirmed by chromophore specific Zn-acetate staining as well as an absorption and fluorescence emission spectroscopy of T-PE (Figs. 1 and 2B).

This result reflected the pattern of N-PE truncation toward T-PE. Absence of any matches between PMF spectrum of β-subunit (of N-PE) and T-PE (Fig. 5) indicated complete degradation of N-PE β-subunit. Whereas, significant matches between PMF spectrum of α-subunit (of N-PE) and T-PE (Fig. 5) indicated the presence of any sequence/structural motifs in α-subunits as a stopping point of degradation. Altogether, this result inferred that T-PE is made up of a single peptide belonging to α-subunit family and hypothesized to obtain from N-PE by complete degradation of β-subunit and limited truncation of α-subunit, spontaneously.

### 3.4. Role of truncated part in folding and stability

The aim of this experiment was to understand the role of truncated residue in the folding and structural stability of N-PE. For this purpose, we have performed in vitro denaturation and renaturation study using urea as denaturing agent. Due to owing similar absorption maxima, folding/unfolding of T-PE and N-PE can be followed by recording the changes in their absorption maxima at 565 nm. We thus measured the absorption spectrum of both proteins in the range of 250–800 nm as a function of [urea] to check the difference in their structural stability. Fig. 7A and B shows the absorbance spectra of N-PE and T-PE as a function of [urea]. Successive reduction in the absorption property (ε_{565}) of both proteins was observed upon an increase in [urea]. The characteristic absorption at 565 nm of PE is because of the phycoerythrobilin (PEB)–apoprotein interaction, spatial arrangement and the surrounding microenvironment of PEB [42]. Reduction in ε_{565} is mainly due to the loss of PEB–apoprotein interaction and tertiary and quaternary level protein-folding [42]. A significant decrease in ε_{565} of both N-PE and T-PE was observed in the presence of 4–6 M [urea]. However, above 6 M [urea] no significant decrease was observed. This may be due to the decrease in the stringency caused by disruption in balanced hydrophilic–hydrophobic environment [43]. Fig. 7C and D shows the denaturation curves, plots of Δε_{565}
Table 2
Stability parameters of the F-PE and T-PE associated with the urea-induced denaturation at pH 7.0 and 25 °C.

<table>
<thead>
<tr>
<th>Protein</th>
<th>( \Delta G^\circ ) (kcal mol(^{-1}) M(^{-1}))</th>
<th>m (kcal mol(^{-1}))</th>
<th>Cm (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-PE</td>
<td>9.76 ± 0.31</td>
<td>2.02 ± 0.31</td>
<td>4.83 ± 0.12</td>
</tr>
<tr>
<td>T-PE</td>
<td>8.42 ± 0.24</td>
<td>1.78 ± 0.28</td>
<td>4.73 ± 0.27</td>
</tr>
</tbody>
</table>

\( \Delta G^\circ \) and m are mean of errors from the average of three independent measurements.

References


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