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Structural characterization and antioxidant potential of phycocyanin from the cyanobacterium *Geitlerinema* sp. H8DM

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ABSTRACT

We are reporting characterization of phycocyanin (PC), a prime light harvesting pigment, composed of nearly the same molecular weight of alpha- (17.5 kDa) and beta- (18.1 kDa) subunits in *Geitlerinema* sp. H8DM. Phycocyanin was purified using ammonium sulfate and anionic exchange chromatography. The fluorescence emission spectrum of PC was 637 nm when excited over 589 nm, which denoted integrity and functionality of protein structure. PC was depicted as a single subunit of ~18 kDa based on SDS-PAGE. However, similarity between both subunits was proven by two spots on gel using two dimensional gel electrophoresis. MALDI ToF/ToF showed structure similarities with alpha and beta subunits of PC. Urea induced Gibbs free energy denoted that folding and structural stability of this pigment is similar to phycocyanin of other species. Biophysical properties of peptide sequence were analyzed for several parameters and 3D model for its correlation as anti-oxidant. Furthermore, verification of *in-silico* data of PC was done by *in vitro* anti-oxidant assays. Hence, due to high availability, stability and antioxidant properties it can be used for auxiliary applications as colorant and therapeutic agent against oxidative stress.

1. Introduction

Cyanobacteria are Gram-negative and extremely adaptable photo-autotrophic prokaryote that directed to evolution of the diverse and richer source of photosynthetic pigments and biometabolites with specific roles [1–5]. *Geitlerinema*, filamentous cyanobacterium, has been considered as an effective carbon sequester [6,7]. Cyanobacteria contain high amount of phycobiliproteins (PBPs) that are major metabolic products associated with light-harvesting complex in photosystem, called as phycobilisome (PBS). The PBS consists of core and rods assembly which contain allophycocyanin (APC; λ_{max}: 650–655 nm) as a core surrounded by C-phycocyanin (C-PC; λ_{max}: 610–620 nm) and occasionally C-phycoerythrin (C-PE; λ_{max}: 540–570 nm) in rods [8]. C-PC is an abundant light-harvesting pigment present up to 20% of the total proteins [9,10]. They usually occur in the heterodimeric form (αβ) and take up an imperative role in energy-transfer cascade by funneling the light-energy toward reaction-center of the photosystems. Assembly of the PC is commenced by the docking of α- and β-subunits that are partially homologous in amino acid sequences (25–40%) but are highly homologous in a three-dimensional structure. Generally, moderate energy absorbing PC occurs as a trimeric (αβ)3 or hexameric ((αβ)3)2 forms along with sole phycocyanobilin attached to the highly conserved region α84- and β84-, β155-cys residues in heterodimer [11]. However, several variants of PBPs have been reported, including single subunit PBPs [12–14]. The studied cyanobacterium was found plenteous in PC production based on PBPs profiling and explored for structural and functional characterization. The PC from *Geitlerinema* sp. was depicted as a single peptide subunit, however, a series of biochemical characterizations revealed the occurrence of α and β subunits of almost similar molecular mass. Moreover, besides the immense role in photosynthesis, several therapeutic properties of several phycobiliproteins including PC have been established, such as antioxidant, anti-inflammatory, anticancer activity and several other applications [15–18]. Hereby, *in silico* analysis using amino acid sequence provides information related to its physical, chemical and functional properties. Elucidation of three-dimensional structures of a protein may provide information about their structural variations toward globally fitted conformations, and physicochemical properties. Hence, the model structure of PC was constructed using computational modeling. 3D model of PC can illustrate the influence of amino acid side chains. In this study, we have detailed *in silico* analysis and homology modeling studies of phycocyanin correlating the contribution of amino acids for functional antioxidant properties.

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2. Materials and methods

2.1. Cyanobacterial growth conditions and identification

The cyanobacterium *Geitlerinema* sp. H8DM was isolated from the sea-shore of Bet-Dwarka, Gujarat, India. The cultures were cultivated in a sterile ASN III growth medium at 28 ± 2 °C under 12/12 h light/dark illumination cycle [19]. The isolated culture was identified through microscopic examination using light microscope (MPN-400, Carl-Zeiss, Germany), Fluorescent microscope (BX-41, Olympus, Japan) and Scanning Electron microscope (Nova NanoSEM 450, FEI Ltd., USA) followed by morphological characterization referring to standard taxonomic keys [20] and monograph. The cyanobacterium was also identified by 16S rRNA gene sequencing using universal primers (8F and 1492R (l)) [21] followed by the phylogenetic tree mapping inferred by maximum likelihood method [22]. Evolutionary analyses were directed using MEGA7 software [23].

2.2. Extraction and precipitation of phycocyanin (PC)

Extraction of PC was carried out using the procedures as described earlier [24], with slight modification. Briefly, the cell biomass of exponentially grown cultures of *Geitlerinema* sp. H8DM was collected by centrifuging the cell suspension at 12,000 ×*g* for 10 min. The biomass was resuspended in extraction buffer (20 mM potassium phosphate buffer: KPB, pH 7) and used for protein extraction by mild sonication at 20 kHz ± 50 Hz for 1–2 min and repetitive freeze (−80 °C)–thaw (20 °C) cycles to lyse the cells. The lysate was centrifuged at 14,000 ×*g* for 20 min at 4 °C to remove chlorophyll and other cell debris. Cell-free suspension of PC was enriched by ammonium sulfate precipitation from 0 to 25% (w/v) with continuous stirring for 1–2 h. The homogenates were centrifuged (14,000 ×*g*, 20 min) and copper-blue colored supernatants were recovered and further precipitated by adding ammonium sulfate to make it up to 45% (w/v) and overnight incubation at 4 °C. The precipitated proteins were collected (centrifuged at 12,000 ×*g*, 15 min) and dissolved it in 20 mM KPB. Protein suspension was dialyzed (Sigma, 12 kDa) against the sample buffer for overnight at 4 °C. Dialyzed protein was further purified by anionic exchange chromatography.

2.3. Purification of phycocyanin (PC)

The Hi-prep anion exchange column of DEAE Sephadex (26/60, GE Healthcare, USA) was used for the purification of PC, based on ionic charge present in the protein. The 1 M NaCl along with 20 mM KPB (pH 7.2) was used as mobile phase, and the flow rate was set at 2 mL/min during the purification process. The column was pre-equilibrated with 20 mM KPB containing 0.5 M NaCl (pH 7.0) followed by injection of 5 mL crude protein (~20 mg/mL). Protein was eluted by step gradient with elution buffer containing up to 0.5 M NaCl. The eluted fractions were monitored by measuring their absorbance at 280 nm. Copper-blue colored fractions rich in PC were opted for further spectroscopic analysis for concentration and purity analysis.

2.4. Biophysical analysis of purified PC

2.4.1. UV–visible spectroscopy

UV–visible spectroscopic analysis of purified PC was performed for a sensitive measure of subtle changes in protein structure and to determine the protein concentration and purity. Absorbance (A) spectra were recorded over 250–900 nm wavelength range by UV–visible spectrophotometer (Specord 210, Analytik Jena, Germany) with 1 cm path length. The purity of PC was determined by calculating the ratio of absorbance at 280 nm and 620 nm (O.D. 620/O.D. 280). Further, the amount of PC in sample was calculated using the following equation [25].

$$\text{PC (mg/mL)} = [\text{O. D } 620 - 0.474\,(\text{O. D. } 650)]/5.34 \quad (1)$$

The amount of PC in sample after purification was also estimated and calculated using Folin-Lowry method [26].

2.4.2. Fluorescence spectroscopy

The fluorescence spectrum of purified PC was measured using a fluorescent spectrophotometer (F-7000, Hitachi High Technologies, Japan) at ambient temperature. The emission band was recorded upon excitation at 589 nm wavelength to check its tertiary structural information. Spectrum was analyzed by software provided by the manufacturer.

2.4.3. PAGE analysis

PC at each successive step of purification was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [27]. Briefly, protein samples were separated on 15% polyacrylamide resolving gel prepared with 4% stacking gel for protein subunit analysis. The standard protein marker ranging from 3.5 to 43 kDa was used to determine the molecular weight (Bangalore GeNei™, India). The resolved gel was stained with zinc acetate staining [28] to visualize the integrity of bilins. Subsequently, silver staining of gel slab was done according to Garfin to visualize peptide pattern based on their molecular weight.

2.4.4. High-performance liquid chromatography (HPLC)

Purified phycocyanin was analyzed for size determination of peptides using high-performance liquid chromatography (HPLC-LC 20AD, Shimadzu, Japan). The 300 µg of protein was heat denatured using the boiling water bath and loaded on a Bio-Sil SEC 125-5 gel filtration column. The mobile phase was 50 mM potassium phosphate buffer (pH 8.0) and flow rate was set at 0.6 mL/min. The column back pressure and temperature were 55 kg/cm^2 and 25 °C, respectively. Protein peaks were detected at 280 nm with 2.0 AUFS. The standard protein marker (ranges 18.4–240 kDa) was run with the same parameters for molecular weight elucidation. The results were interpreted with HP Chemstation software. The molecular weight of PC subunits was calculated using graphs, plotted for KaV *vs.* logarithmic molecular weight of the standard protein marker as described earlier [29,30].

2.4.5. 2D gel electrophoresis

The purified protein was freeze-dried by a lyophilizer (Sentry 2.0, VirTis, SP Scientific, USA) and proceeded using 2D starter kit (ReadyPrep™, Bio-Rad, USA) for isoelectrofocusing (IEF) as described earlier [31,32], with slight modifications. Briefly, 0.2–0.4 mg of lyophilized proteins were dissolved in 150 µL of rehydration buffer and loaded onto a 7-cm immobilized pH gradient (IPG) strip ranges from pH 3 to pH 10 (linear) (Bio-Rad, USA). The strips were incubated in a Protean IEF cell at 20 °C for 16 h active rehydration. The electrophoresis run was initiated as per manual. For the second dimensional run, the strips were equilibrated in equilibration buffer I, followed by equilibration buffer II for 15 min each. The IPG strips were positioned on the top of 15% SDS-PAGE gels and sealed with 0.5% (w/v) agarose. The second dimension separation was performed with a constant current of 100–120 V per gel for 1.5 h. The gels were stained with Coomassie brilliant blue G250 solution and spots were observed for analysis.

2.5. MALDI ToF/ToF

2.5.1. Gel elution and trypsin digestion

Two spots of purified PC resolved by 2D gel electrophoresis were excised and proceeded for digestion by Trypsin-Gold (Promega Corp., Madison, WI, USA). Digested samples were dried and suspended in 0.1% trifluoroacetic acid (TFA) followed by purification using Millipore ZipTips (Sigma Aldrich, USA) with TA buffer (0.1% TFA and acetonitrile - 1:1 ratio) [33].