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Isolation and characterization of thermostable phycocyanin from *Galdieria sulphuraria*

Myounghoon Moon*,‡, Sanjiv K. Mishra,‡, Chul Woong Kim*, William I. Suh**, Min S. Park*,**, and Ji -Won Yang*,**,†**

*Department of Chemical and Biomolecular Engineering, KAIST, 291 Daehak-ro, 373-1, Guseong-dong, Yuseong-gu, Daejeon 305-701, Korea
**Advanced Biomass R&D Center, #2502 Building W1-3, KAIST, 291 Daehak-ro, 373-1, Guseong-dong, Yuseong-gu, Daejeon 305-701, Korea
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Abstract−Phycocyanin is a highly valuable pigmented protein synthesized by several species of cyanobacteria and red alga. In this study we demonstrate the production of thermostable phycocyanin from the unicellular red alga *Galdieria sulphuraria*. Phycocyanin was extracted by repeated freeze-thaw cycles and purified in a two-step process using ammonium sulfate fractionation, at 25% and 50% concentrations. Purified phycocyanin exhibited maximum absorbance at 620 nm, and the purity ratio (A_{620}/A_{280}) was found to be greater than 4. The recovery efficiency of phycocyanin from the crude extract was above 80%. In total, approximately 19 milligram pure phycocyanin was obtained from 3 g of wet cell mass of *Galdieria* sp. Subunits α and β of the protein were separated by SDS-PAGE and analyzed by MALDI-TOF mass spectrometry for identification, which confirmed that the isolated protein is phycocyanin. The molecular weight of α and β subunits of phycocyanin was found to be 17.6 and 18.4 kDa, respectively.

Keywords: *Galdieria sulphuraria*, Red Algae, Value-added Product, Phycocyanin, Purification

INTRODUCTION

Cyanobacteria and red algae are rich in phycobiliproteins that covalently bind to bilins (tetrapyrrole chromophores), and they are important sources of natural pigments used in photodynamic therapies based on their spectral properties. Phycobiliproteins have been classified into allophycocyanin (APCs), phycocyanin (PCs), phycoerythrin (PEs), and phycoerythrocyanins (PECs) [1-3]. Phycobiliproteins are a family of stable and highly soluble fluorescent proteins derived from cyanobacteria and eukaryotic algae [4-6]. These proteins contain covalently linked linear tetrapyrrole groups (metal free) which play a biological role in collecting light through fluorescence resonance energy transfer, and conveying it to a special pair of chlorophyll molecules located in the photosynthetic reaction center [7,8]. The color of phycobiliproteins is due to the presence of covalently bound prosthetic groups that are open-chain tetrapyrrole chromophores bearing A, B, C, and D rings named phycobilins. Phycocyanins have an absorption peak at 615-630 nm and can be excited by orange-red light. PEs are found in most cyanobacterial species and red algae, and have peaks at 530-565 nm green light. APCs usually have an absorption peak at 650 nm of red light. Additionally, chlorophyll a has maximal absorptions at 440 nm and 680-700 nm [9]. They are either blue colored phycocyanobilin (PCB), red phycoerythrobilin (PEB), yellow phycourobilin (PUB), or purple phycobiliviolin (PXB), also named cryptoviolin [10,11]. Phycobiliproteins are classified on the basis of their color into two large groups, the phycoerythrin and the phycocyanin [12].

C-Phycocyanin (C-PC) occurs as the major phycobiliproteins in many cyanobacteria and as secondary phycobiliproteins in some red algae [6,13,14]. The pigment has a single visible absorbance maximum between 615 and 630 nm and a fluorescence emission maximum at around 640 nm. Its molecular weight is between 70,000 and 110,000 Daltons. The pigment is composed of two subunits, alpha and beta, which occur in equal numbers [15].

Phycocyanin serves as a useful fluorescent probe due to its excellent spectral properties, stability, high absorption coefficients, and high quantum yield, which are superior to many synthetic dyes. They are highly soluble in water and exhibit a large Stokes shift, which is very important for detection of the stained material [10,16,26]. Therefore, phycocyanin and related subunits are utilized in a number of applications in foods, cosmetics, biotechnology, diagnostics, and medicine [17-19]. Their fluorescent nature allows them to function as valuable fluorescent tags in highly sensitive fluorescence techniques [2,20]. As a consequence, phycobiliproteins are widely used in clinical and immunological research laboratories [21], where they serve as labels for antibodies, receptors, and other biological molecules in a fluorescence-activated cell sorter, immune-labelling experiments, fluorescence microscopy, and diagnostics. In addition, phycocyanin is non-toxic and poses no health risk whether applied externally or internally. This property makes it useful in various fields of forensic sciences including biotechnology, forensic biology, biotechnology, forensic serology, molecular biology, and recombinant technology. Lastly, due to the aesthetically attractive spectral behavior, phycocyanin is widely used commercially in the dye industry and

†To whom correspondence should be addressed.
E-mail: jwyang@kaist.ac.kr
‡Myounghoon Moon and Sanjiv K. Mishra contributed equally to this work.
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cosmetics. *Spirulina platensis* is one of the major organisms cultivated for the production of phycocyanin [22-25].

Galdieria sulphuraria is an acido- and thermophilic red algae [6,14], which can survive extreme environmental conditions above 40 °C and pH of 1-2. The strain can be cultivated under phototrophic or heterotrophic modes with various types of organic carbon sources. Although *G sulphuraria* is known to be rich in phycocyanin, extraction and purification of phycocyanin from the strain has not been experimentally demonstrated. The aim of the present study is the isolation and characterization of thermostable phycocyanin from *G sulphuraria* using ammonium sulfate precipitation. In particular, the effect of temperature and time on the stability of extracted phycocyanin was scrutinized.

MATERIALS AND METHODS

1. Microbial Culture and Growth Conditions

The red algae *Galdieria sulphuraria* was obtained from the Culture Collection of Algae at the University of Texas at Austin (UTEX, USA). The strain number is designated as UTEX number #2919. The isolation, enrichment, and purification of the culture were carried out using standard microbiological techniques. The microscopic morphology and fluorescence characteristic of culture of *G sulphuraria* is shown in Fig. 1. The culture was cultivated in Cyanidium medium (pH 2.7), which was composed of (per liter) 1.00 g $(NH_4)_2SO_4$, 0.02 g K_2HPO_4, 0.02 g $MgSO_4 \cdot 7H_2O$, and 30 mL soil extract. *G sulphuraria* cells were grown in 500 mL baffled flasks (DURAN®) containing 250 mL of Cyanidium medium under 30-50 μmol photons m^{-2} s^{-1} at 30 °C.

2. Preparation of Phycocyanin Crude Extract

Phycocyanin containing cultures were harvested by centrifugation at 8,000 rpm for 10 min at 4 °C. The cell pellets were washed twice with distilled water. Washed cell mass (3.0 gram wet) was suspended in 30 mL of 100 mM phosphate buffer (pH 7.2) and was freeze thawed in −80 °C and 4 °C. Freeze thaw step was repeated thrice to complete the extraction. The cell debris was removed through centrifugation at 10,000 rpm for 30 min at 4 °C, and blue colored supernatant containing PBP extract was collected in fresh tubes. This extract was termed as a crude extract.

3. Purification of Phycocyanin from Crude Extract

The bluish green crude extract was further purified through fractional precipitation with solid powder of ammonium sulfate. Two cycles of the process were performed, with initial precipitation at 25% saturation followed by second step at 50% saturation via addition of solid ammonium sulfate [27]. Solid ammonium sulfate was dissolved by vortexing and overnight incubation at 4 °C. The precipitate was recovered by centrifugation at 10,000×*g* for 30 min at 4 °C; the clear, colorless supernatant was discarded and the blue precipitate was dissolved in a small volume (10 mL) of 50 mM Na-acetate buffer with pH of 4.5. The quantity (yield) and quality (purity ratio) of the obtained sample was monitored by recording the absorption spectrum from 250 to 800 nm.

4. Spectroscopic Estimation of Phycocyanin

All UV-Vis absorption spectra of crude and purified samples were recorded on a spectrophotometer with a 1 cm path length. In the visible region, phycocyanin and allophycocyanin show maximum absorption at 620 and 652 nm, respectively. The purity of phycocyanin was assessed by calculating the ratio of A_{620} to A_{280}, wherein A_{620} is the maximum absorbance of C-PC, A_{562} is the absorbance of C-PE and A_{280} is the absorbance of total proteins [28]. The absorbance of supernatants containing phycobiliproteins was measured at 620, 652, 562, and 280 nm for calculating the concentrations of phycobiliproteins, using the following equations [29]:

Phycocyanin (mg mL^{-1})=[A_{620}−0.474(A_{652})]/5.34

Allophycocyanin (mg mL^{-1})=[A_{652}−0.208(A_{620})]/5.09

5. Characterization and Identification of Phycocyanin

For a detailed characterization and identification of purported phycocyanin sample, the protein was separated with SDS-PAGE. The resulting two separated bands were carefully excised from Coomassie blue stained gels, divided into small pieces and subjected to in-gel trypsin digestion [30]. Excised gel spots were destained with 100 μL of destain solution (50% methanol) with shaking for 5 min. After removal of the solution, gel spots were incubated with 200 mM ammonium bicarbonate for 20 min. The gel pieces were dehydrated with 100 μL of acetonitrile and dried in a vacuum centrifuge. 50 μL of 10 mM DTT in 0.1 M ammonium bicarbonate was added for shaking incubation at 56 °C for 30 min, and then the sample was

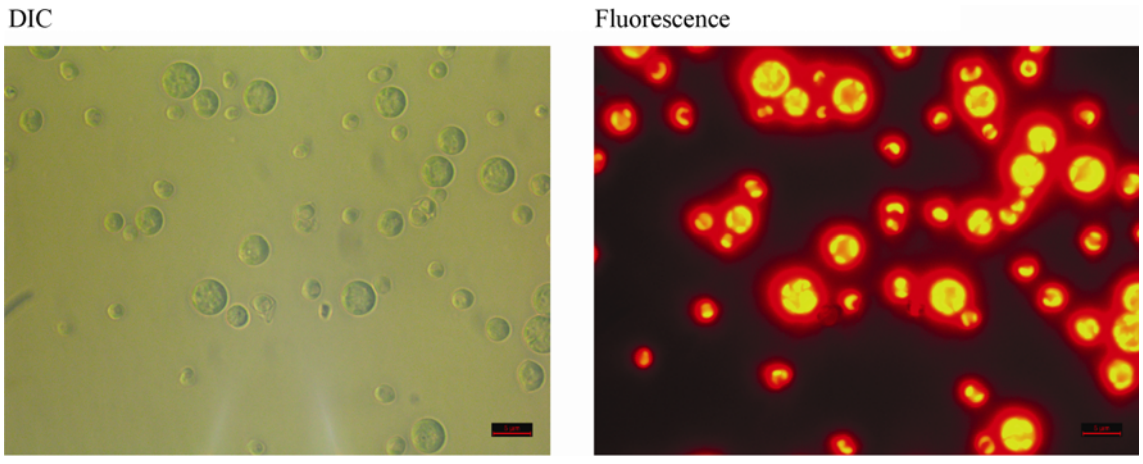

Fig. 1. Differential interference contrast (DIC) microphotographs (left, scale bar: 5 μm) and fluorescent microphotograph (right) of *Galdieria sulphuraria*; the alga shows its unicellular nature and intense fluorescent characters. The fluorescence is caused by phycocyanin.

cooled to room temperature. The supernatant was removed via centrifugation and 200 μL of acetonitrile was added to dehydrate the gel. 50 μL of 55 mM iodoacetamide was added in 0.1 M ammonium bicarbonate and was incubated for 20 min in the dark. Supernatant was removed again via centrifugation; 200 μL of 0.1 M ammonium bicarbonate solution was added for rehydration and 200 μL of acetonitrile for dehydration. The entire rehydration-dehydration process was repeated three times. Finally, the dried gel pieces were digested with 20 μL of 50 mM ammonium bicarbonate containing 0.2 g modified trypsin (Promega) for 45 min on ice. After that the supernatant was removed and replaced with 30 μL of 50 mM ammonium bicarbonate. The digestion was performed overnight at 37 °C. The peptide solution was desalted using ZipTip C18 column (Pierce Co.). The digested protein sample analysis was performed using Ultraflex III Matrix assisted laser desorption Ionization time-of-flight (MALDI-TOF) mass spectrometry measurements.

6. Effect of Different Temperature on Stability of Phycocyanin

To investigate the effect of various temperatures on the stability of phycocyanin, 1 mL of phycocyanin solution in micro centrifuge tube was incubated at 350 rpm in a Eppendorf Thermo mixer comfort (Eppendorf, Hamburg, Germany) for 30 min at different temperatures (25, 35, 45, 55, 62, 65, 75, 85±1 °C). After 30 min the samples were centrifuged to remove any debris and analyzed by spectrophotometer at 620 nm.

To determine the time dependence on thermostability of phycocyanin, the samples were incubated at 62 °C, and the absorbance at 620 nm was measured at regular intervals (0-150 min). The remaining concentration of phycocyanin (C_R, %) relative to the initial concentration was calculated using the following equation [3]. C_R (%)= $C/C_0 \times 100$; the relative concentration of phycocyanin (C_R, %) is the remaining concentration of phycocyanin as a percentage of the initial concentration (C_0).

RESULTS AND DISCUSSION

1. Extraction, Purification and Spectral Analysis of Phycocyanin

The preliminary in-vivo microscopic and spectral analysis of the cells showed that the cells contained high amount of pigments (Figs. 1 and 2). The culture was bluish green and had the absorption peak at 620 nm, which is an indicator for the presence of phycocyanin. For the recovery of any intracellular product, it is necessary to select a proper method for extraction. We selected the freeze thaw method for cell disruption and extraction of phycocyanin. This is the preferred method of choice as it is already widely employed for the extraction of phycocyanin extraction. It has many advantages over other methods, which are not reproducible, low yielding, and could potentially damage the protein's fluorescence properties [31]. All experiments were performed in the dark because phycocyanin is a light- and temperature-sensitive protein.

According to Mishra et al., 2010 [32], phycobiliprotein are most stable near pH 7. Thus, during the extraction of phycocyanin 100 mM of phosphate buffer (pH 7.2) was used to maintain the structural stability of the protein. After the extraction of phycocyanin, the mixtures were centrifuged to remove the unwanted cell debris from the solution. Dark blue crude extract of phycocyanin having an absorption maxima at 620 nm was obtained, and the initial purity ratio was 1.18; the purity ratio of the crude extract isolated using our protocol is higher than that reported in other published articles regarding extraction of phycocyanin from *Spirulina*, *Calothrix* and other species [33,34]. If the initial stage of the process of phycocyanin production, the index of purity is high, the cost of purification will decrease later. Although *G sulphuraria* is unicellular red algae, it incorporates phycocyanin as major phycobiliprotein. Since Phycoerythrin exists in negligible quantities, it does not interfere in the purification process of phycocyanin. The phycocyanin content in *G sulphuraria* has strong specificity to the growth conditions. This demonstrates that the *G sulphuraria* can be a good candidate strain for the purified phycocyanin production.

The first step of ammonium sulfate precipitation starts at 25% saturation, which mainly precipitates unwanted proteins with 91% phycocyanin recovery. The phycocyanin fraction was further precipitated with 50% saturation of ammonium sulfate; this step precipitates all phycocyanin and eliminates other proteins. Precipitated phycocyanin underwent dissolution in sodium acetate buffer (50 mM Na-Acetate buffer pH 4.5). During this step phycocyanin was completely dissolved in buffer, but acetate buffer precipitates linker protein. After this step, the purity ratio reached 4.1. This surpasses high purity analytical grade phycocyanin, which has purity ratio requirement of >4.0 [35,36]. The quantitative assessment of the phycobiliprotein (C-PC, APC, and PE) content was investigated in all the steps of extraction and fractional precipitation (Table 1).

The absorption spectrum of the phycocyanin solution of *G sul-*

Table 1. Determination of spectrophotometric purity of thermo stable phycocyanin from *Galdieria sulphuraria* at each stage of purification

	Crude extract	Fractional precipitation with $(NH_4)_2SO_4$ (25% saturation)	Fractional precipitation with $(NH_4)_2SO_4$ (50% saturation)
Volume of sample	30 mL	30 mL	10 mL
Dilution	1 : 5	1 : 5	1 : 15
652	0.436	0.387	0.314
620	1.032	0.941	0.825
562	0.422	0.383	0.32
280	0.872	0.855	0.199
CPC	0.155	0.142	0.127
Purity index	1.18	1.10	4.14
Total CPC (mg)	23.18	21.27	18.99
Yield (%)	100.00	91.79	81.93

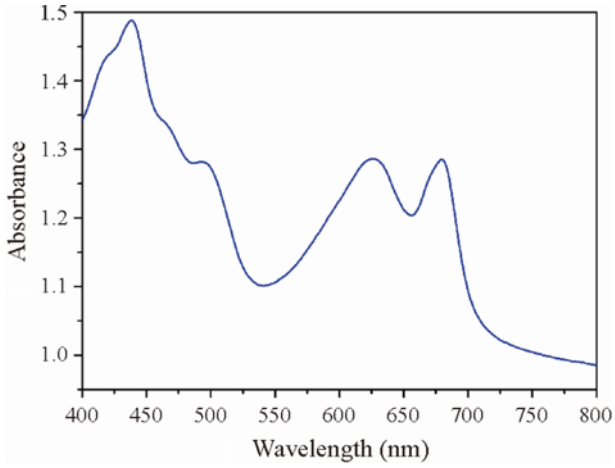

Fig. 2. In vivo absorption spectra of unicellular red alga *Galdieria sulphuraria*. Note the strong absorption by chlorophyll *a* at 680 nm and a second absorption maximum at 620 nm corresponding to phycocyanin. Other absorption peak from 400 to 500 nm is related to carotenoids.

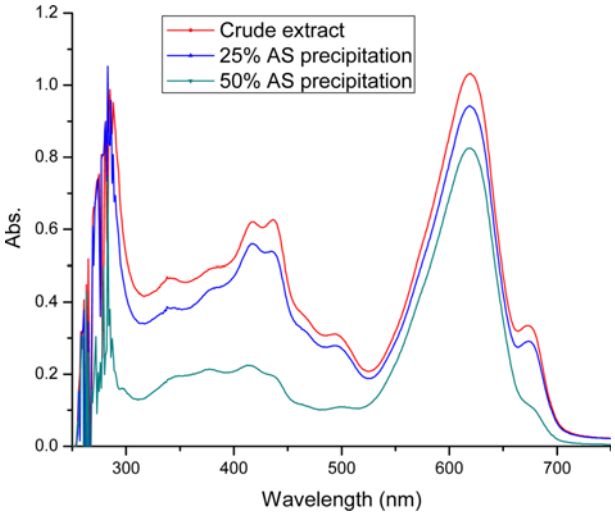

Fig. 3. UV-Vis absorption overlay spectra of phycocyanin from *Galdieria sulphuraria*, at each step of purification. Steps: 1. Crude extracts; 2. 25% saturated ammonium sulfate precipitation, and 3. 50% saturated ammonium sulfate precipitation.

phuraria was acquired by UV-Vis spectrophotometer. The spectrum ranged from 250 to 750 nm at each step presented in Fig. 3. The in vivo (Fig. 2) crude extract (Fig. 3) of unicellular red alga has the typical "sharp-peak" of phycocyanin with absorption maxima at 620 nm. Blue colored culture and such absorption spectrum characteristics are typical in other phycocyanin producing strains such as *Spirulina platensis*, *Phormidium*, and *Calothrix* sp. etc [2, 34,35,37-39].

CPC-A
MKTPITEAIAAADNQGRFLSNTELQAVNGRYQRAAASLEAARSLTSNAERLINGAAQAVYSKFPYTSQMP
GPQYASSAVGKAKCARDIGYYLRMVTYCLVVGGTGPMDEYLIAGLEEINRTFDLSPSWYVEALNYIKANH
GLSGQAANEANTYIDYAINA LS

CPC-B
MLDAFAKVVAQADARGEFLSNTQLDALSKMVSEGNKRLDVVNRITSNASAIVTNAARALFSEQPQLIQPG
GNAYTNRRMAACLRDMEIILRYVSYAIIAGDSSILDDRCLNGLRETYQALGVPGASVAVGIEKMKDSAIAIA
NDPSGITTGDCSALMAEVGTYFDRAATAVQ

Fig. 4. N-terminal amino acid sequences of α and β subunits of phycocyanin.

2. Identification of α and β Subunits of Phycocyanin by MALDI-TOF Analysis

The α and β subunits bands separated by SDS-PAGE were identified via MALDI-TOF mass spectrometry (Fig. 4). Spectra were collected from 500 shots per spectrum over *m/z* range 600-5,000 Da. The search program MASCOT, developed by Matrix Science (http://www.matrixscience.com/), was used for protein identification by peptide mass fingerprinting. The resulting peptide mass fingerprint was blasted in the National Centre for Biotechnology Information (NCBI) database using MASCOT Search engine. Protein molecular masses and isoelectric points (pI) of the identified subunits are presented here (Table 1). MALDI-TOF-TOF confirmed that the subunits of the purified protein belong to the phycocyanin.

3. Effect of Different Temperature on Stability of Phycocyanin

In the last few years, there has been growing interest in the possible usages of phycocyanin in food coloring, natural dye, fluorescent applications, nutraceuticals, and pharmaceuticals. Stable phycocyanin has been produced chemically and by protein engineering, and new purification procedures allow highly pure phycocyanin to be obtained at high yields [2]. But the application of phycocyanin in food, dye, cosmetics, fluorescence study, and other applications is narrow due to its sensitivity to temperature, light, and air, which results in the loss of its intense blue color. There are many chemical and additives which can extend the stability of phycocyanin, but many are toxic and thus cannot be used for human consumption [3].

When the phycocyanin solution was incubated for 30 min in a temperature range from 0 to 95 °C, the absorption of the spectra remained consistent until 60 °C, with steady reduction in absorbance at 620 nm after that point. This is due to the alteration in the protein from its native conformation to a denatured one. The heat induced denaturation on phycocyanin is an irreversible process. The denaturation of protein increased from 65 to 95 °C. The stability of phycocyanin also decreased quickly as the temperature was elevated from 75 to 95 °C. In addition, to study the kinetics of the denaturation process the phycocyanin solution was incubated at 62 °C for an extended time, and the UV/VIS absorption spectra were also examined periodically as a function of time. After 150 min at 62 °C, the C_R value of the phycocyanin solution decreased down to approximately 65% of the initial value.

Table 2. Identification of α and β subunits of phycocyanin by MALDI-TOF

Separation by	Protein	Subunit	Protein mol mass (Da)	Protein (pI)	No. of peptide identified	AA sequence
PAGE (SDS)	Phycocyanin	α	17609	5.81	5	162
		β	18412	4.96	8	172

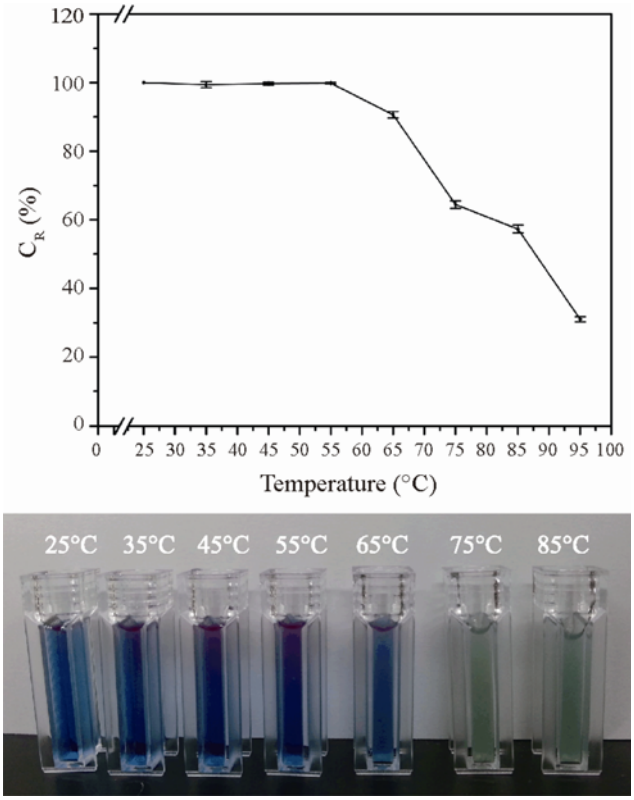

Fig. 5. Effect of different temperature on the C_R value of phycocyanin solution (reaction time: 30 min).

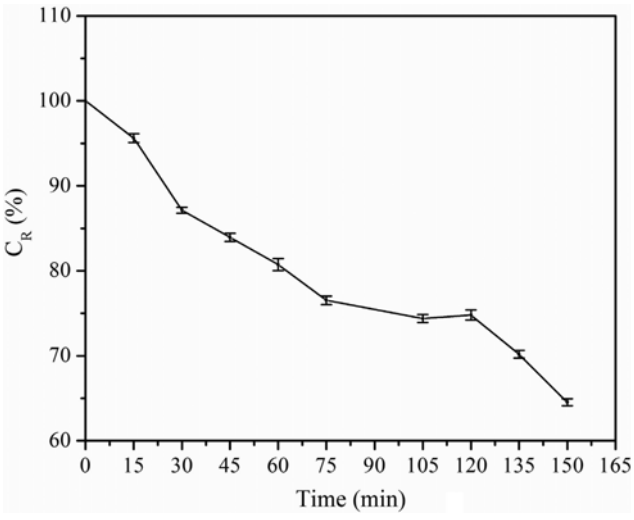

Fig. 6. Effect of different time on the C_R value of phycocyanin solution (temperature: 62 °C).

This shows the phycocyanin from this alga is thermostable until 60 °C. In contrast, Chaiklahan et al. 2012 [3] demonstrated that 47 °C is the critical temperature for the stability of phycocyanin from *Spirulina* sp., with a sharp drop in the C_R and half-life values at higher temperatures. Therefore, the thermal characteristics of *Galdieria sulphuraria*'s phycocyanin are better than that of phycocyanin from other strain. The past studies show that the thermophiles can survive at higher temperature, but that the photosynthetic organisms have upper limits of survival at 73 °C [40]. The production of thermostable phycocyanin in this strain can be considered as an evolutionary adaption to a hotter environment, and therefore this strain is a promising candidate for the production of thermostable phycocyanin.

CONCLUSION

We isolated and characterized a thermostable phycocyanin from red algal unicellular strain belonging to *G. sulphuraria*, which exhibits its ability to remain stable at high temperatures up to 60 °C, and we established a method for the efficient recovery of phycocyanin. The yield recovery of thermostable phycocyanin was 81.93% with purity ratio (A_{620}/A_{280}) of 4.14. Both α and β subunits were characterized by MALDI-TOF analysis, which confirmed that the protein is phycocyanin. The α and β subunits were 17.6 and 18.4 kDa molecular mass, respectively. The fact that phycocyanin from this species maintains stability at higher temperature makes it more commercially competitive over phycocyanin from other species.

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