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Research Article

Extraction and Purification of Phycoerythrin-A Natural Colouring Agent from *Spirulina Platensis*

Suresh P.Kamble[1]*, Ganesh P. Vikhe[1], Datta R. Chamle[2]

[1]Centre for Biotechnology, Pravara Institute of Medical Sciences (Deemed to be University) Loni-Bk 413736, Ahmednagar (MS) India
[2]Department of Botany, Sharada Mahavidyalaya, Parbhani-431401 (MS) India

***CORRESPONDING AUTHOR**
Suresh P. Kamble, Centre for Biotechnology, Pravara Institute of Medical Sciences (Deemed to be University) Loni-Bk 413736, Ahmednagar (MS) India.

Email: kamsuresh@gmail.com

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ABSTRACT
Phycoerythrin (PE) is a natural colouring agent found in blue green algae, such as *Spirulina platensis* and is used as natural colour agent in food and beverage industries. In the present study, PE was extracted from *Spirulina platensis* by freezing and thawing method and purified by ammonium sulphate precipitation followed by anion exchange chromatography using DEAE cellulose and acetate buffer. Recovery of PE after ammonium sulphate precipitation was 80.25% with purity of 2.60 and after chromatography; it was 56.94% with purity of 4.74. Purified PE showed absorption maxima at 565 nm that is the characteristic of C-PI group of phycoerythrins. Electrophoretic analysis revealed that the purified PE has two subunits viz α and ß with molecular weight of 17 kDa and 19 kDa respectively.

KEYWORDS: *Spirulina platensis*; Phycoerythrin; Ammonium sulphate precipitation; Anion exchange Chromatography; SDS-PAGE

INTRODUCTION

Cyanobacterium (Blue green algae) *Spirulina platensis* is valuable due its richness in proteins and vitamins and thus being used as health food and therapeutic agent [1]. Cyanobacteria and algae possess a wide range of colored compounds, including chlorophyll, carotenoids and phycobilliproteins [2]. Spirulina contains many accessory photosynthetic pigments and one of the most important pigments is Phycobilliproteins [3] which is involved in efficient energy transfer chain in photosynthesis. It is a hydrophilic, brilliantly colored and stable fluorescent pigment protein that can be classified into three main groups: Phycoerythrin (λ_{max}~565 nm, deep red), Phycocyanin (λ_{max}~620nm, deep blue) and Allophycocyanin (λ_{max}~650nm, bluish green) [2]. A fourth Phycobilliprotein known as Allophycyanin B has also been shown to be present in lower amounts [4, 2]. Glazer, 1981; Samarakoon and Jeon, 2012 [5-6] demonstrated that Phycoerythrin transferred the absorbed light efficiently to chlorophyll via phycocyanin, and Allophycocyanin. Most cyanobacterial Phycobiliproteins are composed of two different kinds of polypeptides of which one is light (α, MW- 12-19 kDa) and other is heavy (ß, MW-14-21 kDa). These chains are generally present in equimolar amounts [7] and are generally organized as trimetric (αß) 3 discs but larger aggregates like hexameric (αß) 6 discs are also

found [8]. The brilliant colors of Phycobiliproteins are mainly due to covalently bound prosthetic groups that are open-chain tetrapyrrole chromophores bearing A, B, C and D rings named phycobilins. These phycobillins are phycocynobilin that is blue colored, phycoerythrobillin-red color, phycourobilin-yellow colored and purple colored phycobiliviolin. Glazer (1985) [9] showed that these chromophores are bound to polypeptide chain at conserved positions. Cyanobacterial Phycobiliproteins have gained considerable importance in the commercial sector, as they have several applications. Phycierythrins are currently widely used in the production of food and cosmetics, and play an important role in many biochemical techniques due to their fluorescence properties [10]. PE is commonly used as a fluorescent label in immunology, cell biology [11] and flow cytometry [12]. It is also applied as a natural food dye [13], broad range pharmaceutical applications and as a marker in gel electrophoresis and iso-electrofocusing [14]. Phycoerythrin is the most widely used Phycobiliproteins in fluorescent probes and has quantum yields of 82-98% [15].

Hence, the present study was focused on optimization of a purification process to obtain high purity Phycoerythrin from *Spirulina platensis*.

MATERIALS AND METHODS

Materials

All chemicals used in the present study were of analytical grade. *Spirulina platensis* was obtained from NB Laboratories Pvt. Ltd., Nagpur, India. Dialysis membrane (Dialysis membrane-70, MWCO- 12-14 kDa) was procured from HiMedia (India) and Protein Marker was obtained from Bangalore GeNei, India.

Algal strain and cultivations

The cyanobacterium *Spirulina platensis* was obtained from NB Laboratories Pvt. Ltd., Nagpur, India. Algae were grown in semicontinuous culture for 15 days with easy culture medium containing sodium carbonate, sodium nitrate, potassium biphosphate and fertilizer containing N:P:K at 16:16:16 with a content of 8.5, 1.5, 0.5 and 0.6 gram/l, respectively.

Extraction and Estimation of Phycobilliproteins

A 500 mL of homogenized log phase (14 days old) culture was centrifuged at 6000 rpm to obtain pellet. The pellet was suspended in 100 mL of 20 mM Acetate buffer containing 50 mM Sodium Chloride and 0.002 M Sodium Azide (pH 5.10) as described by Kumar et al. 2014 [16]. Repeated freezing at -20°C and thawing at room temperature till the cell mass becomes greenish was used for the extraction of phycobilliproteins. Crude extract was prepared by removing cell debris at 6000 rpm for 10 minutes.

Phycoerythrin content was measured as described by Bennett and Bogorad (1973) [17] and purity was determined using the formulae

Purity = OD_{562}/ OD_{280}

Purification of PE

Obtained crude extract was kept for 65% ammonium sulphate precipitation at 4°C for overnight. The pellet was recovered by centrifugation at 5000 rpm for 15 minutes at 4°C and dissolved in 10 mL of the same extraction buffer. Then it was dialyzed against the extraction buffer using Dialysis membranes (Dialysis membrane-70, MWCO- 12-14 kDa) procured from HiMedia (India). Dialysis was performed twice against 1000 mL extraction buffer fist at room temperature and then at 4°C for overnight. The resultant extract was recovered from the dialysis membrane and filtered through 0.45 µm filter (Sartorious).

A 25cm×2cm column of DEAE-Cellulose was prepared for purifying the Phycoerythrin protein. Column was equilibrated with 150 mL of Acetate buffer (pH 5.10). 10 mL of dialyzed and filtered sample was placed on the top of the DEAE-Cellulose column with the help of a syringe. Then, 50 mL of Acetate buffer (pH 5.10) was then applied to the column to wash out any unbound protein. The bound proteins and thus phycobilliproteins were eluted with a linear gradient of acetate buffer in the pH range from 3.76-5.10 and 5 ml fractions were collected at a flow rate of 20 ml/hr.

Determination of absorption spectra

Absorption spectra were determined by scanning the sample in a range of 300-750 nm wavelengths by UV-2450 spectrophotometer (Shimadzu).

SDS-PAGE analysis of PE

Electrophoresis of purified PE in polyacrylamide gel was carried out in a vertical chamber using 12% polyacrylamide gel with SDS (SDS-PAGE) [18]. Molecular weight of the purified protein was determined by Protein Marker obtained from Bangalore GeNei, India, along with the sample. Following electrophoresis, the gel was stained by 0.1% Coomassie Brilliant Blue G250 solution.

RESULTS

Phycocyanin- important cyanobacterial accessory pigment having wide industrial applications is mainly obtained from Spirulina. But very few reports are available for the extraction of phycoerythrin from *Spirulina platensis*. Many reports are available for the purification and characterization of PE from various algal strains [19-23], only few are from India [24-27].

The extraction of C-PE wad carried out in four steps as, Step I-crude extract preparation, Step-II-ammonium sulphate precipitation (65%), Step III- dialysis and finally Step IV- purification by DEAE cellulose. It is evident from Table 1 that these steps are very efficient to obtain high purity and recovery of PE. Precipitation of Phycobiliproteins with 65% saturated ammonium sulphate resulted in 85.81% recovery with a purity of 2.80, although there was no significant increase in purity after dialysis (Table 1). In ion exchange chromatography PE with maximum purity was obtained. The efficiency of the method was shown by 7 times increase in the purity of purified PE compared with crude (Fig. 1). The absorption spectra of the purified PE showed a prominent peak at 565 nm (Fig. 2). Noticeably there was no peak corresponding to phycocyanin or allophycocyanin. SDS-PAGE analysis of the purified PE revealed two bands of 17 kDa and 19 kDa corresponding to α and ß subunits respectively (Fig. 3).

Table 1: Purification table of Phycoerythrin from *Spirulina*

Steps	Volume (ml)	PE (µg/ml)	Purity of PE (OD_{565}/OD_{280})	Percentage of recovery of PE
Crude extract	100	45	0.64	100
Ammonium sulphate precipitation	10	384	2.59	79.35
Dialysis	10	299	2.61	72.71
Column chromatography (DEAE Cellulose)	5	374	4.57	57.79

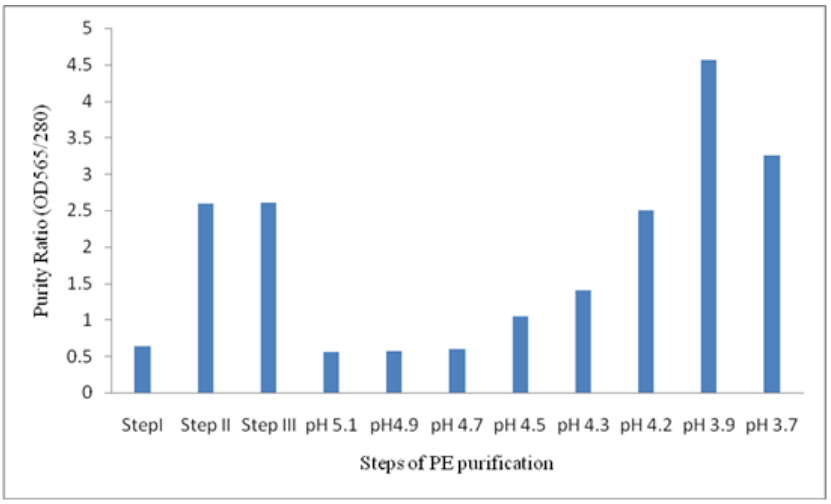

Fig. 1 Purity of Phycoerythrin attained at various steps viz. Step I- crude extract, Step II-Ammonium sulphate precipitation, Step III- Dialysis and elution of the column using acetate buffer with a linear gradient of pH from 3.7 to 5.1

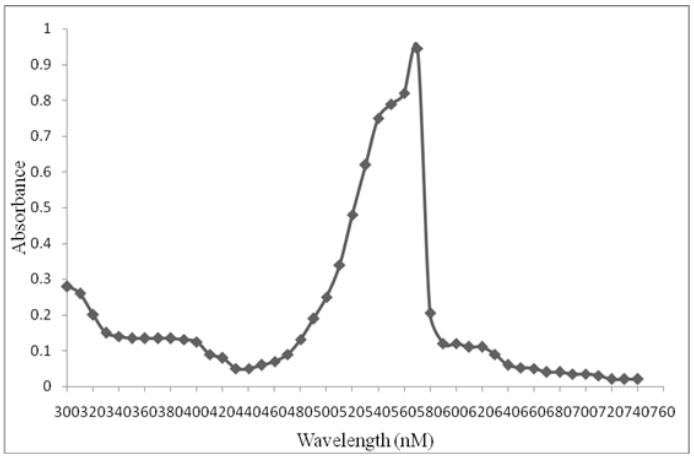

Fig. 2: UV-Vis Spectrophotometric spectra of purified Phycoerythrin

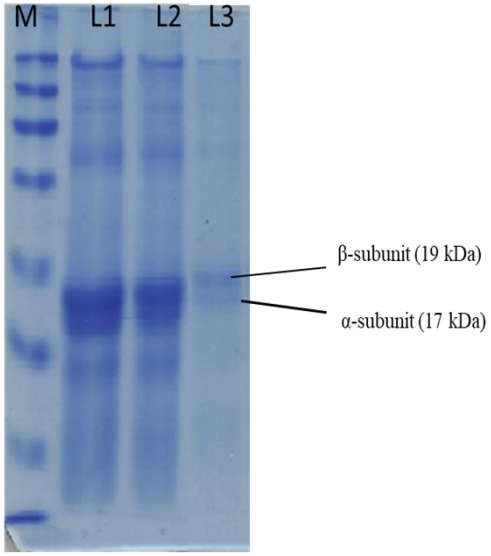

Fig. 3: SDS-PAGE analysis of purified Phycoerythrin

M- Protein marker (Myosin 205 KDa, Phosphorylase b 97.4 KDa Bovine serum albumin 66 KDa, Ovalbumin 43 KDa, Carbonic anhydrase 29 KDa, Soyabin trypsin inhibitor 20.1 KDa, Lysozyme 14.3 KDa, Aprotinin 6.5 KDa, Insulin 3.5 KDa.), L1- 65% ammonium sulphate precipitated sample, L2- Dialyzed sample, L3- Purified PE

DISCUSSION

Due to fluorescent property, PE is widely used in food and cosmetics industry as immunodiagnostics and as analytical tool. PE is also particularly appropriate for use as a protein marker in such electrophoretic techniques as SDS-PAGE, or isoelectric focusing and size-gel exclusion chromatography and probably could be used as alimentary or cosmetic colorant [14]. Obtaining pure PE is challenging because of its limited distribution and available methodologies particularly in cyanobacteria. In this study, following 65% ammonium sulphate precipitation, we have achieved a recovery of 85.81% with a purity of 2.81 which is greater than that reported by others. Ranjitha and Kaushik (2005) [24] reported 85% recovery of phycoerythrin with a purity of 2.89 after 55% ammonium sulphate precipitation from *Nostoc muscorum* while around 80% recovery of PE content with a purity ratio of around 1.5 for young and old cultures from three cyanobacteria viz. *Phormidium sp*. A27DM, Lyngbya *sp*. A09DM and *Halomicronema sp*. A32DM has been after the treatment of crude extract with 70% ammonium sulphate [26]. It is

pertinent to mention here that the purity achieved after ammonium sulphate precipitation is high enough for using the phycoerythrin in foods and feeds. Natural colorants producing industries may help the production of PE by scaling up of this process. During chromatographic separation a purity of 4.95 was achieved which is higher or comparable to that reported by Tchernov *et al.* (1999) [28]. Similar result was reported by Tripathi *et al.* (2007) [25] from Lyngbya *arboricola* using step wise purification with a purity of 5.25. The recovery (62.5%) was however not as high as reported by others [24, 26]. On the other hand, the whole process described in this study involving single step chromatographic separation is simple and comparable or better than the other existing methods for purification of Phycoerythrin as it does not involve any additional purification steps like membrane filtration, gel filtration or use of organic solvents [25, 28-29]. C-PE generally exists in hexameric state i.e. (αß) 6. The α and ß subunits vary with their originations in molecular mass from 15 kDa to 22 kDa. Galzer and Cohen-Bazirre (1971) [30] reported α and ß subunits of 20 kDa and 22 kDa from Aphanocapsa sp. (strain 6701) while Bennett (1972) [31] reported α and ß subunits of 18.3 kDa and 20 kDa from *Fremeyella diplosiphon*. Ranjitha and Kaushik (2005) [24] also reported α and ß subunits of 19.4 kDa and 16.5 kDa from *Nostoc muscorum*. The molecular mass of the α (16 kDa) and ß (18 kDa) subunits obtained in this study are in compliance with the earlier reports. Cyanobacterial phycoertythrin or C-PE is generally divided into two subtypes viz. C-PE I and C-PE II. The most common C-PEI exhibiting absorption spectra with maximum absorbance near 565 nm [25]. C-PE-II from Synechococcus strains WH8020 and WH8103 had absorption maxima at 495 and 543, and 492 and 543,respectively [32]. Chakdar and Pabbi (2012) [27] purified PE from *A.variablis* (CCC421) and showed that the absorbance maxima is 565 nm that is the characteristic of group I phycoerythrin. Amidst a large array of natural products produced by cyanobacteria, phycobiliproteins seem to be most colourful and attractive components due to their potential biotechnological and industrial application. Therefore the uses of this pigment for human benefit need the economic process development and the selection of suitable cyanobacterial culture.

CONCLUSION

In conclusion, we have shown that blue green algae *Spirulina plantensis* is a superior source of C-PE-a natural colouring agent with a purity value 4.57. This study may help the researchers and entrepreneurs to develop strategies for large scale production of phycoerythrin. However, toxicological studies must be carried out to assess their biotechnological feasibility for commercial production.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest in this research article.

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