Sustainability, Agri, Food and Environmental Research, (ISSN: 0719-3726), 11(X), 2023:
http://dx.doi.org/10.7770/safer-V11N1-art655

Phycocyanin from a cyanobacterium of Sundarbans.

Ficocianina de una cianobacteria de Sundarbans

Pinaki Hazra[1], Sahana Ghosh[1], Gargi Saha Kesh[2]

[1]State Aided College Teacher, Department of Microbiology, Vijaygarh Jyotish Ray College, Kolkata 700032, West Bengal, India

[2]Associate Professor, Department of Microbiology, Vijaygarh Jyotish Ray College, Kolkata 700032, West Bengal, India. ORCID 0000-0002-7797-0633

ABSTRACT

Cyanobacteria comprise of a varied bacterial phylum that are responsible for the oxygenation of the environment. Cyanobacteria perform 10 to 25 % of total photosynthesis. The main photosynthetic accessory pigments in cyanobacteria are phycobiliproteins. One of the different types of phycobiliprotein is Phycocyanin (λ_{max}at 620 nm). Cyanobacteria possess not only antioxidant protection, but it has been seen to be responsible for anti-inflammatory activities. World's one of the biggest mangrove forests is Sundarbans in the Bay of Bengal. We have isolated phycocyanin from a cyanobacteria strain, AP9F (GenBank accession number FJ847844). Ammonium sulphate was used for sequential precipitation of crude extract. Increased purity ratio of phycocyanin was achieved through dialysis of the protein sample, anion exchange chromatography using a DE 52 column and Sephadex G-100 column chromatography. The protein consists of two subunits of α (20 kDa) and β (25 kDa) each in $(\alpha\beta)_2$ dimer association. The molecular weight of purified PC was found to be 90 kDa. Antioxidant activity was shown by the purified CPC. Increase in PC concentration decreased the rate of haemolysis. We hereby suggest that the phycocyanin isolated is quite similar other phycocyanin from various freshwater as well as marine cyanobacteria.

Key words: Sundarbans; cyanobacteria; phycocyanin; antioxidant activity.

RESUMEN

Las cianobacterias forman parte de un filo bacteriano variado que es responsable de la oxigenación del medio ambiente. Las cianobacterias realizan entre el 10 y el 25 % de la fotosíntesis total. Los principales pigmentos accesorios fotosintéticos de las cianobacterias son las ficobiliproteínas. Uno de los diferentes tipos de ficobiliproteína es la ficocianina (λmaxat 620 nm). Las cianobacterias no sólo poseen protección antioxidante, sino que también se ha visto que son responsables de actividades antiinflamatorias. Uno de los bosques de manglares más grandes del mundo es Sundarbans en la Bahía de Bengala. Hemos aislado ficocianina de una cepa de cianobacteria, AP9F (número de acceso de GenBank FJ847844). Se utilizó sulfato de amonio para la precipitación secuencial del extracto crudo. Se logró una mayor proporción de pureza de ficocianina mediante diálisis de la

muestra de proteína, cromatografía de intercambio aniónico usando una columna DE 52 y cromatografía en columna Sephadex G-100. La proteína consta de dos subunidades de α (20 kDa) y β (25 kDa), cada una en asociación de 2 dímeros (αβ). Se encontró que el peso molecular de la PC purificada era de 90 kDa. La CPC purificada mostró actividad antioxidante. El aumento de la concentración de PC disminuyó la tasa de hemólisis. Por la presente sugerimos que la ficocianina aislada es bastante similar a otras ficocianinas de varias cianobacterias de agua dulce y marinas.

Palabras clave: Sundarbans; cianobacterias; ficocianina; actividad antioxidante.

Significance Statement

This is the first detailed report on the photosynthetic pigment, phycocyanin of a cyanobacteria isolated from Sundarbans. The molecular weight of purified C-PC was determined. Antioxidant and anti hemolytic property was shown by the C-PC.

INTRODUCTION

Cyanobacteria are well representation of their hardy group, which help them to survive to unusual genetic adaptations that protect the organisms [1]. They are thought to be the first organisms to carry out oxygenic photosynthesis. These organisms can be found in a wide range of habitats including soil, marine and freshwater environments. Extreme habitats like hot spring water, arctic and antarctic environments are also inhabited by cyanobacteria [2]. Cyanobacteria dominate and constitute the greater part of microbial mats as primary producers of the microbial foodweb. They carry out a plant-like oxygenic photosynthesis in which two photosystems (PS II and PS I) are coupled in series and there lies their uniqueness [3]. A collection of chromo proteins, soluble in water and photosynthetic pigments such as chlorophyll a, beta carotene, myxoxanthphylletc make the phycobilisome. In cyanobacteria the main photosynthetic accessory pigments found are phycobiliproteins.

In these cells major phycobiliproteins found are phycoerythrin (PE, absorbance maxima at 540-570 nm,and emission maxima at 570-590 nm,phycocyanin (PC, Absorbance maxima at 610-620 nm and Emission maxima at 645-653 nm) and allophycocyanin (APC, absorbance maxima at 650-655 nm and Emission maxima at 657-660 nm) [4]. Phycocyanin (PC) is the major component of phycobiliprotein. Cyanobacteria while growing in extreme weather conditions can reduce the salt content and promote levels of C, N, and P including moisture content of the salt affected soils and thus can be exploited for the amelioration of the salt affected soils [5]. PC is used to capture light energy for photosynthesis, unique to cyanobacteria and is responsible for the distinctive deep blue colour. Besides showing antioxidant protection, it is also responsible for anti-inflammatory and hepatoprotective activities [6]. In addition to this, PC (Phycocyanin) selectively inhibits cyclooxygenase-2 (COX-2) activity [7]. In vitro research studies shows that PC reduces cellular oxidative damage by scavenging alkoxyl, hydroxyl and peroxyl radicals and reacts with peroxynitrite and hypochlorous acid. Additionally Blue-Green algae are also having notable amounts oligo-and polysaccharides, water-soluble phenolic compounds, PUFAs (Polyunsaturated fatty acids), among which many are

known to show remarkable antioxidant and anti-inflammatory characteristics [8]. There are 2 subunits of PC α and β, these subunits α (low mw polypeptide) and β (high mw polypeptide) subunits are associated in trimers $(αβ)_3$ and hexamers $(αβ)_6$ [9]. 140-210 kDa is found to be the approximate molecular weight of PC [10]. The techniques that are used for purification of PC involve density gradient centrifugation, ammonium sulphate precipitation, ion exchange chromatography and gel filtration chromatography.

A mangrove forest like Sundarbans are now is the focus of scientific interests. It isrecognized worldwide for the rich source of biodiversity, considering salinity to be a determinant. Sundarbans is one of the largest mangrove forests in the world, situated in a unique bioclimatic zone within a typical geographical location in the coastal region of the Bay of Bengal. It is a sight of age old legacy bestowed with wonderful picturesque splendor and natural resources. Cyanobacterium AP 9F; FJ847844, our strain was isolated from Sundarbans. The16S rRNA gene sequence of the cyanobacteria AP 9F was submitted to GenBank and accession number was found to be FJ847844.The dendrogram of the isolated cyanobacteria AP 9F showed species richness on the basis of morphological, biochemical, and physiological characteristics. This strain is taxonomically associated to LPP group[11].

MATERIALS AND METHODS

Chemicals: ASN III Media components (AR grade) i.e. sodium chloride, magnesium sulfate, magnesium chloride, sodium nitrate, di potassium hydrogen phosphate, calcium chloride, potassium chloride, sodium carbonate, citric acid, ferric ammonium citrate, EDTA, vitamin B_{12} , A 5 trace minerals and DE 52, Standard Protein Marker have been purchased from Himedia, India. Sephadex G-100 was procured from Sigma and AAPH {2,2'-Azobis(2-amidinopropane) dihydrochloride} was purchased from Merck.

Culture Conditions: The marine cyanobacteria culture was cultured in ASN III Media in 12 hour light and dark cycle at 25 °C, with intermittent shaking.

Extraction of Phycocyanin:

After one month of incubation, the cell mass of cyanobacterium 9F was collected. The cell mass was washed with distilled water repetitively and then 5 gm of freeze-dried cell mass was homogenized by Homogenizer (Remi, India). 100 ml of 20 mM Tris-HCl buffer (pH 6.5), 10 mM EDTA was used to resuspend homogenized cells. The cell suspension was incubated with 1 mg/ml hen egg white Lysozyme for quick rupture of the cell wall. Sonicator (Piezo- U-Sonic, India) was used for sonication for 60 secs. The cell mass was incubated for 4 hours at room temperature after sonication. Then it was subjected to repetitive freeze-thawing at -20 °C and 4 °C. Appearance of reddish blue colour was considered as the release of phycobiliprotein. Centrifugation at 10000 g for 30 minutes at 4 ^{o}C was done with the mixture. Clear supernatant was then collected.

Spectroscopic Measurements:

The collected supernatant was taken for measurement of absorbance by UV –VIS spectrophotometer (LI-295UV Visible, Intech). It showed a distinguishing peak at 620 nm. The ratio of absorbance at 620 nm and 280 nm (A_{620}/A_{280}) was measured and purity was calculated. At 620 nm, 652 nm, 562 nm, PC, APC and PE showed maximum absorbance respectively.

The concentration of PC, APC and PE can be calculated with the help of the following equations:

PC (mg/ml) = [A_{620} -0.0474 (A_{652})]/5.34,

APC (mg/ml) = [A_{652} -0.208(A_{620}]/5.09,

PE (mg/ml) = [A_{562}- 2.41(PC) – 0.849(APC)]/9.62

PC Purification:

At 10 ˚C, the entire PC purification procedure of was carried out. To achieve 25 % ammonium sulphate saturation, in 100 ml of crude extract of the sample,18.75 gm solid ammonium sulphate was gradually added and kept at 4 ˚C, overnight. Centrifugation was done at 1000 g for 30 minutes at 4 ˚C (Eppendorf Centrifuge 5430R). 50 % saturation was achieved with the addition of 37.5 gm solid ammonium sulphate and kept at 4 ˚C for 24 hours. The resulting solution was centrifuged at 1000 g for 30 minutes. The pellet mainly comprising of PC was dissolved in 100 ml acetate buffer (0.1 M, pH 4.5). Then the suspension was centrifuged at 10000 g for 30 minutes. The pellet containing mainly basic proteins was discarded. The resultant supernatant was brought to ammonium sulphateprecipitationto achieve 50 % saturation and kept overnight at 4 °C. In 10 ml of 20 mM Tris-HCl buffer (pH 8.1), the precipitated PC was dissolved and then it was dialyzed for 10 hrs at 4 ˚C against the same buffer. During the dialysis process, the buffer was changed twice for complete removal of ammonium sulphate.

In anion exchange chromatography 20 mM Tris-HCl buffer (pH 8.1) was used to pre-equilibrate the DE 52 column. The column was loaded with dialyzed sample and washed with ten bed volume of 20 mM Tris-HCl buffer (pH 8.1). Elution was done first with 10 bed volume of 0.05 M NaCl solution and later by addition of a range of concentrations of NaCl (0.1 M, 0.15 M, 0.2 M and 0.25 M) in 20 mM Tris-HCl buffer (pH 8.1)and the sample was then collected in 2 ml micro centrifuge tubes. Flow rate of the sample was set at 0.5 ml/min. Scanning of all fractions was done in the range of 250 nm to 800 nm. Samples with purity ratio of A_{620}/A_{280}> 3.5 was then pooled together. The resultant solution was brought to 50 % ammonium sulphate saturation. In 10 ml 20 mM Tris- HCl buffer (pH 8.1) the pellet was resuspended and then dialyzed against water at 4°C. Then the sample was freeze – dried.

Gel Filtration:

Gel filtration chromatography was performed to determine the molecular weight of the native protein. 10 mM potassium phosphate buffereded was used to pre-equilibrate the Sephadex G-100 column. Calibration was done using egg albumin (molecular weight 45 kDa), bovine serum albumin (molecular weight 67 kDa) and bovine γ-globulin (molecular weight 158 kDa).

Electrophoretic Methods:

SDS-PAGE:

SDS-PAGE (1.5 mm thick) was performed according to Laemmli[3] using a 15 % and 5 % polyacrylamide as resolving and stacking gel respectively. Samples were dissolved in sample buffer of equal volume containing 2 % (w/v) SDS, 10 % (v/v) glycerol, 5 % (v/v) β-mercaptoethanol, 0.002 % (w/v) bromophenol blue and 60 mM Tris-HCl (pH 6.8), and then boiled for 8 minutes. Electrophoresis was performed at room temperature. Coomassie brilliant blue (G 250) staining was carried out to visualize proteins. Pre-stained protein ladder (MBT092 from Himedia) was taken as reference.

Native PAGE:

In Native PAGE proteins while moving through the gel matrix get separated on the basis of the molecular properties such as size, shape and charge. Native PAGE was performed using a 10 % and 5 % polyacrylamide as resolving and stacking gel. Tris – HCl (pH 8.8) was used as running buffer. Coomassie brilliant blue (G 250) staining was carried out to visualize proteins.

Zinc Acetate Staining:

Zinc acetate staining was used to observe the fluorescent property of the extracted protein of cyanobacteria. At room temperature, the native gel without stain was incubated for 5 minutes with 20 mM of zinc acetate solution [12]. Then the fluorescence was observed under UV light.

Antioxidant Activity:

Preparation of RBC suspension:

Blood samples were collected from healthy volunteers in heparin coated vials. RBCs were separated by centrifugation at 1000 g for 10 minutes. Phosphate buffered saline was then used to wash the samples thrice. RBCs were then resuspended in the same buffer to achieve 5% haematocrit level.

Measurement of Haemolysis:

In this experiment, RBC suspension in phosphate buffered saline was taken as a control. Different concentrations (10 µM – 150 µM) of phycocyanin(PC) were mixed with RBC suspension and incubated for 15 minutes at 37˚C. The mixed samples were then incubated with 50 mM AAPH and subjected to haemolysis for 3 hours.

After that, an aliquot of 1ml sample was taken and centrifuged at 3000 g for 2 minutes. To determine the extent of haemolysis, the absorbance of supernatant was measured at 540 nm [12]. RBC was incubated with distilled water and its absorbance was considered as absorbance of 100% haemolysis.

2.10.3. Effect of AAPH in PC Solution:

The extract of 50 µM PC was incubated with AAPH for 40 minutes. Thus the spectral changes were recorded between 300 nm to 800 nm at each 10 minutes interval [13].

RESULTS AND DISCUSSION

The quantitative analysis of the phycocyanin was summarized in Table 1. The spectra of UV- Visible absorption of each step of purification were shown in Fig – 1. SDS-PAGE in Fig-2(a) and native PAGE in Fig-2(b) and Fluorescent property of PC were given in Fig 3. The results of the effect of AAPH in PC solution and antioxidant activity of PC were shown in Fig-4 and Fig-5 respectively.

Table 1: Quantitative evaluation of PC from 5 gm dry weight of cells, at various stages of purificatioN

Purification step	Purity ratio A_{620}/A_{280}	Separation factor A_{620}/A_{652}	PC (mg)	Recovery of PC (%)
(1)Crude extract	0.877	1.993	0.327	100
(2)Ammonium sulphate precipitation with 25% saturation	1.799	2.24	0.35	96
(3)Ammonium sulphate precipitation with 50% saturation	2.12	2.48	0.36	82
(4)DE 52	3.37	3.75	0.382	35

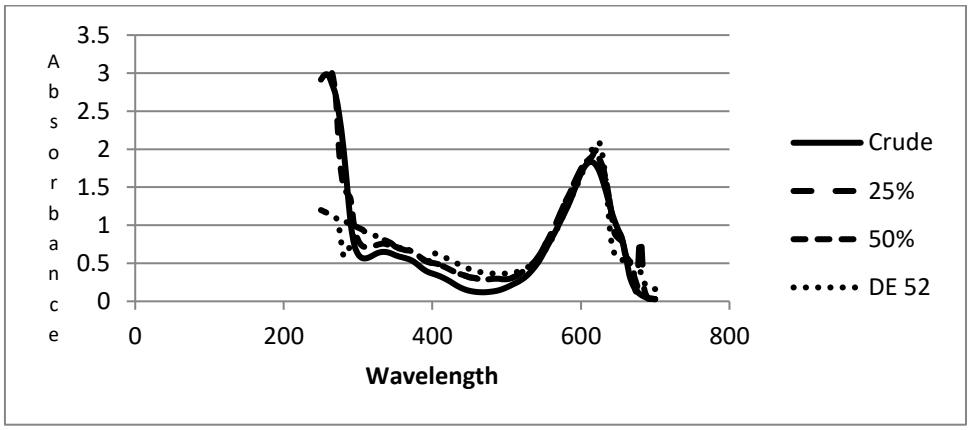

Fig-1. Overlay absorption spectra of PC at each stage of purification

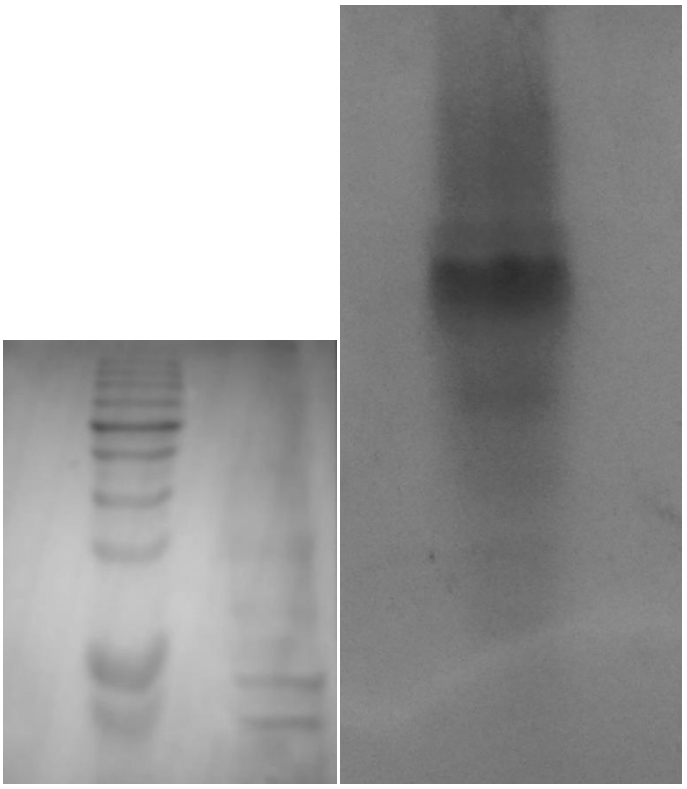

Fig-2.(a). SDS PAGE of C-phycocyanin: lane 1: molecular marker, lane 2: Purified PC, (b) Native PAGE of PC

Fig-3. Fluorescence emission by phycobiliprotein containing PC

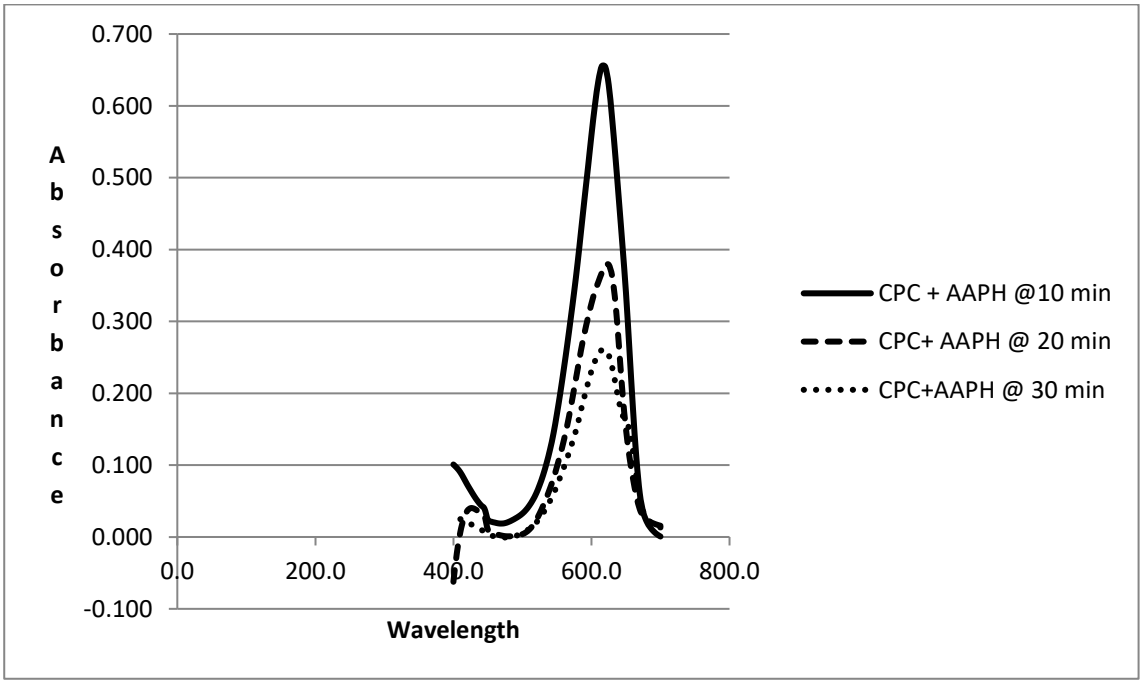

Fig-4. Spectroscopic measurement of PC incubated with AAPH in time dependent manner

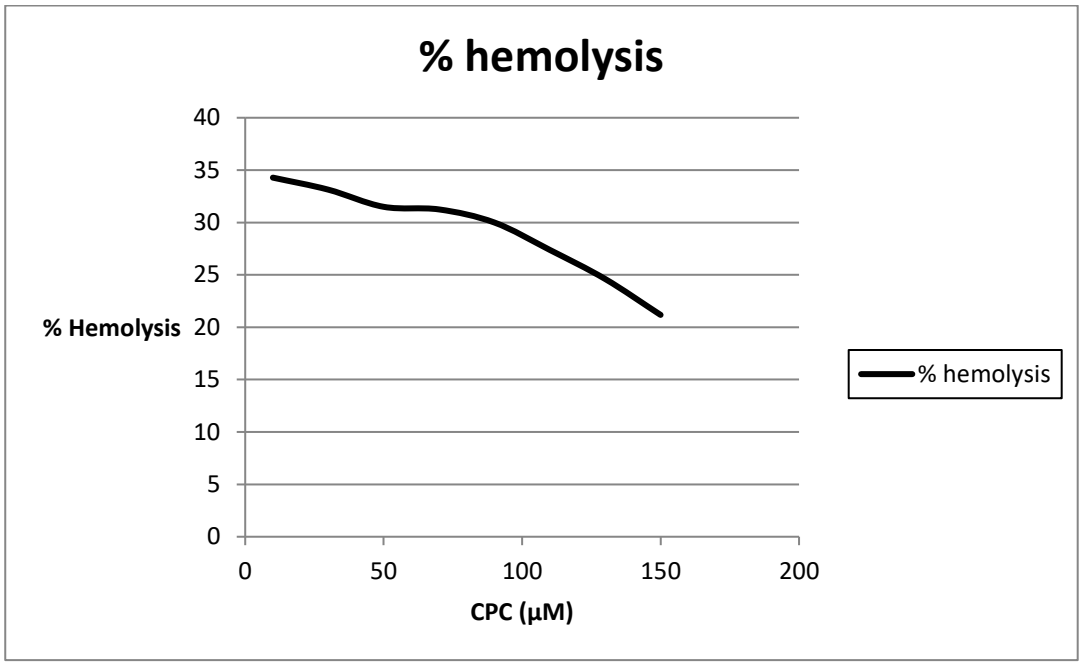

Fig-5. Percentage of haemolysis in human RBCs pre-incubated with different concentrations of PC and then subjected to haemolysis by 50 mM AAPH for 3 hours

Phycocyanin Extraction: AP 9F cell mass was harvested from the medium and was washed with 20 mM Tris-HCl buffer (10 mM EDTA, pH 6.5). Then 5 gm of freeze-dried cell mass was homogenized by Homogenizer (Remi, India). The homogenized cell was resuspended in 100ml of 20 mM Tris-HCl buffer [10 mM EDTA, pH 6.5 and lysozyme (1 mg/ml)]. After sonication, the cell mass was incubated at room temperature for 4 hours. Then it was subjected to repeat freeze-thawing at -20°C and 4°C. anddark cobalt blue supernatant was collected and cell debris was removed.

Phycocyanin Purification: Sequential precipitation of the crude extract of the supernatant was carried out twice by using ammonium sulphate to achieve 25 % and 50 % saturation respectively. Pellets (25 % ammonium sulphate saturation) contain no PC but contain other unwanted proteins which can be evidenced by spectroscopic measurements. After 50 % ammonium sulphate saturation, precipitated PC was collected and dissolved in acetate buffer to precipitate basic proteins. After completion of dialysis PC was loaded on DE 52 column. The column was pre-equilibrated with 20 mM Tris-HCl buffer (pH8.1). The column was loaded with dialyzed sample and 10 bed volume of 0.05 M NaCl solution was used for elution. Later, the elution was done by adding different concentrations of NaCl. The recovery of the eluted PC was 35 % with purity ratio (A_{620} /A_{280}) of 3.37.The quantitative evaluation of the phycocyanin content are summarized in Table 1.

Spectroscopic Measurement: Purity ratio (A_{620}/A_{280}), separation factor (A_{620} /A_{280}) and absorption spectrum of phycocyanin increased in each step of purification (Fig-1). High separation factor is due to low contamination of Allophycocyanin.

Estimation of molecular weight: Figure 2 indicates that, PC is composed of two subunits α and β with molecular weights of 20 kDa and 25 kDa respectively. The PC protein subunits are in the type $(\alpha\beta)_2$ .In Sephadex G-100 Gel filtration Chromatography, Bovine Serum Albumin or BSA elutes before purified PC and PC elutes before bovine gama globulin . As molecular weight of BSA is 67 kDa and that of bovine gama globulin is 158 kDa, the molecular weight of native purified PC can be determined. From these it can be confirmed that the molecular weight of PC could be 90kDa (2α 20 kDa each and 2β 25 kDa). Presence of a single band on native- PAGE (Fig 2 b) confirms purity and subunit integrity of PC.

Observation of Zinc Acetate Staining: Presence of fluorescence indicates that PC contains bilin-linked polypeptide (fig-3)

Observation of Antioxidant Activity: Figure 4 shows that PC exhibit maximum absorbance at 620 nm but when AAPH was added in PC solution, there was a progressive decrease of absorbance of PC at 620 nm.

As PBS is isotonic to human RBC, when RBC was incubated with PBS buffer at 37° C for 3 hrs, negligible haemolysis was observed. But when AAPH, a water soluble free radical generator, added to the solution, it induces oxidative haemolysis of RBC suspension. On the other hand as after the addition of PC in the solution, the rate of haemolysis is reduced. Therefore, the rate of haemolysis decreases with increasing concentration of PC. The result is shown in Fig- 5.

Cyanobacteria are one of the most genetically wide-ranging set of organisms ever studied. They can perform oxygenic photosynthesis. Marine cyanobacteria AP9F was isolated from sea water of Sundarbans. AP 9F is one of the few strains isolated and studied from Sundarbans [10]. The properties of AP9F are not so well documented. This study is mainly focused on separation, efficient purification of PC and detection of antioxidant activity of the purified phycocyanin. Initially cells were homogenized and freeze thawed at - 20° C. After that, the cell lysate was precipitated eventually with 25% and 50% saturation of ammonium sulphate to precipitate PC. Acetate buffer was used to dissolve isolated PC pellet and eliminate some basic protein which increased purity ratio (A_{620} /A_{280}) of PC. Then the precipitated PC was dialyzed overnight to get rid of salts and low molecular weight proteins. In concluding purification process dialyzed sample was loaded on DEAE- cellulose column. PC was eluted between 0.15 M and 0.25 M NaCl concentration. Purity ratio of the eluted PC (A_{620} /A_{280}) increased to 3.37. Ultimate purification was done by SDS-PAGE. The molecular weight of purified PC was 90 kDa which consist of two subunit α (20 kDa) and β (25 kDa) in a dimer association $(\alpha\beta)_2$.

Antioxidant activity measurement using AAPH was done after purification of PC. AAPH reduced the absorbance of PC at 620 nm in a time dependent manner, which caused disappearance of blue color of PC. With varying concentration of PC, it was found that the rate of haemolysis decreased when PC concentration was

increased. The concentration of PC used for haemolysis experiment was in parity with other PCs used in antioxidant experiments [14].

We here report the purification of a photosynthetic dye from a cyanobacteria isolated from Sundarbans. Our strain (cyanobacterium AP 9F; FJ847844) is taxonomically associated with LPP group. The report contributes significantly to the pool of information involving diverse flora and fauna of the mangrove forest. The result suggests this dye molecule is quite similar to other phycocyanin molecules found in different cyanobacteria, when the molecular weight and subunit composition are concerned.

AP9F shows similarity with phycocyanin from other cyanobacteria with respect the antioxidant activity of phycocyanin. In future, extensive studies need to be carried out about different aspects of this dye and other bioactive compounds present in this cyanobacterium.

CONCLUSION

The rate of haemolysis decreases with increasing concentration of PC. It can be concluded from the result that this protein possess antioxidant properties and is quite similar to other PC molecules present in different other cyanobacteria in respect to molecular weight and subunit composition.

Acknowledgement:

The authors thank Prof. Joydeep Mukherjee, School of Environmental Studies, Jadavpur University, Kolkata, India for providing the cyanobacteria strain AP9F.On behalf of all authors, the corresponding author states that there is no conflict of interest.

Funding

University Grants Commission, Government of India (F.No. 42-217/2013 (SR) dated 22.03.2013).

Studies in humans and animals

There was no study involved with humans and or animals.

REFERENCES

Udomluk S, Peter R H, Colin B, Yuwadee P (2005) The distribution of cyanobacteria across physical and chemical gradients in hot springs in northern Thailand. FEMS Microbiol Ecol 52:365–376.

Nyok-Sean L, Minami M, Amirul Al-A A (2015) Cyanobacteria: Photoautotrophic microbial factories for the sustainable synthesis of industrial products. BioMed Res Int 1-10.

Laemmli U K (1970) Cleavage of structural proteins during the assembly of head of bacterial phage T4. Nature 277: 680-685.

Lucas J S (1995) Physiological ecology of cyanobacteria in microbial mats and other communities. New Phytol 131(1): 1-32.

Singh J S, Kumar A, Rai A N, Singh D P (2016) Cyanobacteria: A precious bio-resource in agriculture, ecosystem, and environmental sustainability. Front. Microbiol 7:529.

Shukkur M F, Nithin B B, Devarajan A, Shamala D S (2014) C-Phycocyanin confers protection against oxalate-mediated oxidative stress and mitochondrial dysfunctions in MDCK cells. PLoS One 9(4): e93056.

Roche-Nagle G, Connolly E M, Eng M, Bouchier-Hayes D J (2004) Antimetastic activity of a cyclooxygenase-2 inhibitor. Br J Cancer 91: 359–365.

Romay C, Gonzalez R, Ledon N, Remirez D (2003) C-phycocyanin: A biliprotein with antioxidant, anti-inflammatory and neuroprotective effects. Curr Protein PeptSci 4: 207-216.

Williams V P, Glazer A N (1978) Structural studies on phycobiliproteins. J Biol Chem 253 (1):202-211.

Hazra P, Saha Kesh G (2017) Isolation and purification of phycocyanin from cyanobacteria of a mangrove forest. Appl Biol Chem 60: 631.

Pramanik A, SundararamanM, Das S, Ghosh U (2011) Isolation and characterization of cyanobacteria possessing antimicrobial activity from the Sundarbans, the world's largest tidal mangrove forest. J. Phycol47(4):731-43.

Berkelman T R, Lagarias J C (1986) Visualization of bilin-linked peptides and proteins in polyacrylamide gels. Anal Biochem 156(1): 194-201.

Romay C., Gonza´lez R (2000) Phycocyanin is an antioxidant protector of human erythrocytes against lysis by peroxyl radicals. J Pharm Pharmacol 52: 367–368.

Sanaa MM, Soha SM Mostaf, Shanab Emad A Shalaby , Ghada I Mahmoud (2012) Aqueous extracts of microalgae exhibit antioxidant and anticancer activities. Asian Pac J Trop Biomed 2 (8): 608-615.

Received: 10th October 2022; Accepted: 11th Jule 2023; First distribution: 18th September 2023.