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Full length research paper

Isolation and binding affinity of C-phycocyanin to blood cells and genomic DNA as well as its diagnostic applications

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In the analytical field, many of the chemicals used as fluorescent probes are dangerous and expensive. The use of natural flourochromes like C-phycocyanin (C-PC) in diagnostics is being looked upon as a replacement for organic flourochromes. In this study, C-PC was extracted from algal biomass by six different methods, and analyzed for its binding affinity towards red blood cells of human and fowl blood, platelets and lymphocytes of humans, and genomic DNA of human and plant. The comparative staining ability of C-PC and the commonly used ethidium bromide stain towards the genomic DNA was finally analyzed. The freezing and thawing method resulted in a maximum C-PC yield and the algal biomass dried under shadow by air circulation gave the higher purity. The maximum binding affinity of C-PC was observed with human lymphocytes at 25°C and a C-PC dilution ratio of 1:10^4. In case of fowl red blood cells, the maximum affinity was observed at 25°C with dilution ratio of 1:10^5. The comparative study on the staining ability of ethidium bromide and C-PC towards the genomic DNA revealed a more or less similar strength of reaction. Thus, it is proposed that the partially purified natural dye, C-PC, could be used as a substitute of ethidium bromide. It may be applied as a fluorescent reagent for immunological analysis.

Key words: C-phycocyanin, blood cells, DNA staining, fluorescent microscopy, *Spirulina.*

INTRODUCTION

In the quest of revealing nature's secrets during past centuries, science has explored many concealed natural resources, thus opening a big market to the chemical industries. The chemicals used in the early ages have biohazards, expensive and are carcinogenic in nature such as ethidium bromide. So it was desired to find out new chemicals which are economical and safe to handle. C-PC is an antenna pigment found in many cyanobacteria and eukaryotic algae to increase efficiency of photosynthesis by collecting light energy at wavelength over which chlorophyll absorbs poorly (Glazer, 1981; Wehrmeyer, 1990). It exhibits a variety of pharmacological properties such as antioxidant, anti-inflammatory, neuroprotective and hepatoprotective (Romay et al., 2003). C-PC contain multiple chromophore prosthetic groups, which are responsible for the fluorescent properties of these proteins. It belongs to the family of phycobiliprotein that are well suited as a fluorescent reagent without any toxic effect for immunological analysis since they have a broad excitation spectrum and fluorescence with a high quantum yield (Hardy, 1986; Glazer, 1994; Kulkarni et al., 1996). C-PC is commonly used as natural dyes in food and cosmetics and replaced the synthetic dyes. Phycocyanin has a significant antioxidant, anti-inflammatory, hepatoprotective and radical scavenging properties; even it is used in food and cosmetics, as they are nontoxic and non-carcinogenic (Romay and Gonzalez, 2000). The use of natural flourochromes like phycobiliproteins in diagnostics is being looked upon as a replacement for organic flourochromes. C-PC is a stable protein and does not alter its spectral characteristics in the staining process of tissues and DNA samples. In view

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of the above, the present study deals with the objective to develop a standardized protocol for extracting C-PC from its natural sources, and to analyze the binding affinity of C-PC towards different cells and genomic DNA. The study will be useful in diagnostic purposes and to protect the environment from contamination with synthetic toxic dyes.

MATERIALS AND METHODS

Microbial culture and growth conditions

The mixed cultures of different *Spirulina* species were utilized for the isolation of C-PC. The algae were grown in batch culture under different growth conditions. The bacterial culture was inoculated in media containing $NaHCO_3$ (4.5 g/l), K_2HPO_4 (0.5 g/l), $NaNO_3$ (1.5 g/l), NaCl (1 g/l), K2SO4 (1 g/l), $MgSO_4.7H_2O$ (0.2 g/l), $FeSO_4.7H_2O$ (0.01 g/l) and $CaCl_2.2H_2O$ (0.04 g/l). The culture was maintained at 30±2ºC with an illumination of 3-5 K.lux light intensity provided by cool white fluorescent tubes with a dark and light cycle of 12:12 hours (Venkataraman et al., 1982). The growth of mixed algal mass was monitored spectrophotometrically at 560nm. All the experiments of drying and extraction were carried out in triplicates.

Biological samples

About 10 ml of intravenous human blood sample were collected from the 4 healthy people from Hayes Memorial Hospital of SHIATS, Allahabad and stored at 4ºC for further use. The Fowl blood samples were collected from a local chicken shop. The leaf samples of guava cultivar were collected from the Orchard of the Department of Horticulture, SHIATS, Allahabad. The young leaves were washed, wrapped in aluminum foil and stored at -80ºC until further use.

Extraction of C-PC from mixed culture of *Spirulina* sp.

In the present study, six different extraction methods (three wet biomass and three drying methods) were tried to optimize C-PC isolation. Out of six, the best method resulted in the maximum C-PC recovery was selected. Simultaneously, the isolation of C-PC from blue green algae was preceded with the optimized isolation protocol. The concentration and purity of C-PC was calculated by the method of Boussiba and Richmond (1979) and Bennett and Bogorad (1973), respectively. The algal biomass was determined by measurement of absorption at 560nm in a spectrophotometer. C-PC was extracted from the wet biomass by using following methods.

Water extraction: Harvested biomass of *Spirulina* was suspended in distilled water and kept at 4ºC for 24 hours. The extract was centrifuged at 6000 rpm for 10 minutes and the supernatant was subjected to phycocyanin estimation.

Homogenization of cells: Harvested biomasss was homogenized using homogenizer at 4ºC for 5 min in presence of phosphate buffer at pH 7.0. The extract was centrifuged at 6000 rpm for 10 min. and then the supernatant was subjected to phycocyanin estimation.

Freezing and Thawing: The wet biomass was frozen at -20ºC and thawed at 4ºC repeatedly for 4 hours in 10 ml of phosphate buffer at pH 7.0, the extract was centrifuged at 6000 rpm for 10 min, and the supernatant was subjected to phycocyanin estimation.
The harvested biomass of *Spirulina* was subjected to different drying methods and subsequently followed by incubation at 4ºC for 24 hours in phosphate buffer at pH 7.0 (0.1 M). The following methods are used.

Drying in water bath: One gram of wet biomass of *Spirulina* was transferred to beaker and kept in water bath at 50ºC for one hour. The dried biomass was then ground in mortar using pestle and sieved through 120-mesh size sieve.

Drying in direct sun light: One gram of wet biomass was dried in direct sun light for one hour, when ambient temperature was 35ºC. The dried powder was then ground in mortar using pestle and sieved through 120-mesh size sieve.

Air-drying: One gram of wet biomass was dried at 25ºC under air circulation for 1 hour in shadow (without exposure of direct sunlight). The dried powder was then ground in mortar using pestle and sieved through 120-mesh size sieve.

Binding affinity of C-PC to human blood cells

The binding affinity towards human blood cells (i.e., the anucleated cells viz., RBCs and platelets) has been investigated at different incubation temperatures. Human blood cells were isolated by the method of Bhatia (1977). The isolated cells (5%) were incubated with serially diluted C-PC for 30 minutes in the dark at different temperature (20, 25 and 37ºC). The stained RBCs were then washed with isotonic phosphate saline buffer to remove the excess stain. A drop of these stained red blood cells was mounted on the slide with the mounting

Table 1: Comparisons of different wet methods for extraction of C-PC

Extraction Methods	Yield of C-PC (mg/g)	Purity ratio (A_{620}/A_{280})
Water Extraction at 4ºC	13.40±1.1	0.40±0.1
Homogenization at 2ºC	82.10±0.8	0.59±0.03
Frozen at -20ºC and thawed at 4ºC repeatedly for 4h	86.30±1.1	1.32±0.05

medium (9 parts glycerol + 1 part distilled water) and then observed for fluorescence through Nikon fluorescent microscope.

Binding affinity of C-PC to nucleated cells

The nucleated cells analyzed include the lymphocytes isolated from human blood (Yadava and Mukherjee, 1992) and the fowl red blood cells isolated from fowl blood samples (Badakere et al., 1982). The cells (10%) were incubated with the serially diluted C-PC for 30 minutes in the dark at different incubation temperatures (20, 25 and 37ºC) temperature. The stained human lymphocyte cells / fowl red blood cells were then washed separately with 0.01mM PBS, pH 7.2 to remove the excess stain. A drop of these stained cells were separately mounted on the slide with the mounting medium and observed the fluorescence.

Binding affinity of C-PC to genomic DNA

The binding affinity of C-PC was analyzed with human genomic DNA isolated from human blood (Bangalore Genei DNA isolation Kit,) as well as with plant genomic DNA isolated from guava leaf tissues (Porebski et al., 1997)). The isolated DNA samples were further purified by treating with RNase and phenol-chloroform-isoamylalcohol (Sambrook et al., 1989). The purified DNA was quantified by spectrophotometric method, stained with C-PC and separated by agarose gel electrophoresis that was observed under gel documentation system. The lowest concentration of C-PC to stain the genomic DNA was analyzed by serially diluting C-PC at different concentrations.

Comparison of staining ability of C-PC with ethidium bromide

For elucidation of the staining ability of C-PC as a nuclear stain, the genomic DNA was stained with C-PC and ethidium bromide separately at different concentration and run the test samples on a 0.8% agarose gel and the DNA bands were visualized under gel document system. The staining performance of both the stain compounds was analyzed at different dilutions.

RESULTS

Extraction of C-PC from mixed culture of *Spirulina* sp.

C-PC was extracted by different wet and dry methods. Their efficiency was determined by C-PC concentration and its purity ratio. The data obtained from the various observations were statistically analyzed by using analysis of variance.

Extraction from the wet biomass

In the water extraction method the cells shows yield of C-PC 13.4 mg/g and purity ratio 0.40. In the homogenization method it was 82.10 mg/g with purity ratio of 0.59. In wet biomass freezing at -20 and thawing at 4ºC repeatedly for 4 hours yielded a higher C-PC concentration of 86.3 mg/g with purity ratio 1.32 (Table 1). Correlation techniques were employed to study the relationship between yield and purity of C-PC. The correlation coefficient obtained between the above two variables was 0.686 which showed positive correlation i.e., as the yield of C-PC due to different methods increased, simultaneous increase in the purity of C-PC was also observed (Figure 1).

Extraction by drying methods

In water bath drying method, the C-PC obtained with concentration and purity of 16.5 mg/g and 0.90, respectively. In drying method of biomass by sun light the C-PC obtained with concentration of 64.8 mg/g and purity ratio of 0.82. The yield of C-PC from the air-drying method with concentration of 80.0 mg/g and the purity ratio 1.78 was observed (Table 2). The correlation coefficient was 0.684 which showed positive correlation between the two variables. That is, as the yield of C-PC due to different methods increased, simultaneous

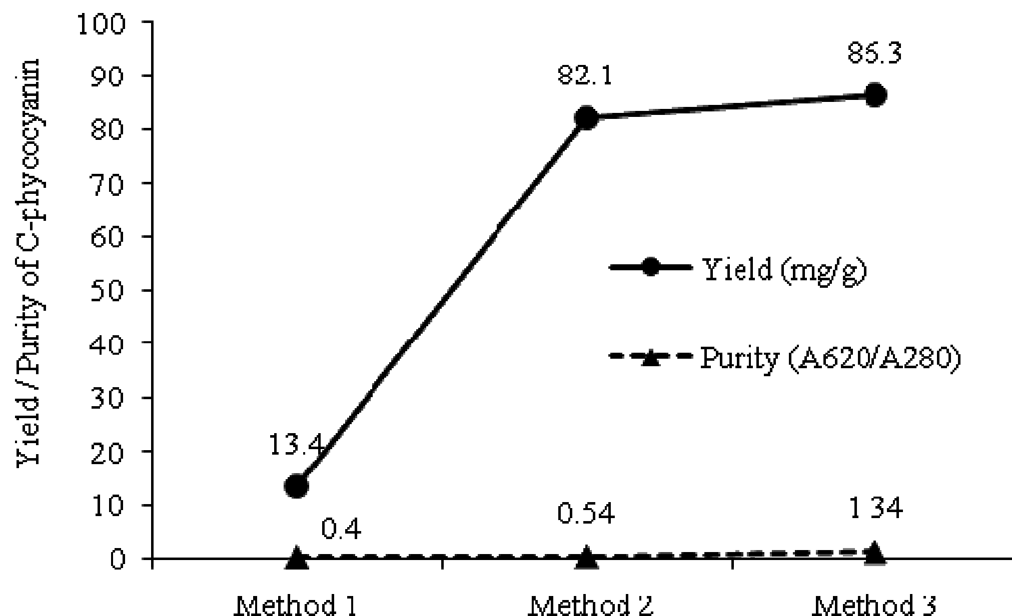

Figure 1: Correlation coefficient between the variables of yield and purity of C-PC by different wet methods (Method 1: Water extraction, Method 2: Homogenization of cells, Method 3: Freezing and thawing)

Table 2: Comparison of different drying methods for extraction of C-PC

Drying methods	Drying Temp.	Yield of C-PC (mg/g)	Purity ratio (A_{620}/A_{280})
Water bath	50ºC for 1h	16.5 ± 0.8	0.90 ± 0.07
Sun dried	Ambient temp. was 35ºC ± 2ºC for 1h	64.8 ± 1.71	0.82 ± 0.03
Air dried	By air current at 25 ± 2 ºC for 1h	80.0 ± 1.9	1.78 ± 0.06

increase in the purity of C-PC was also observed as shown in Figure 2.

Binding affinity of C-PC to blood cells

The binding affinity of C-PC to the anucleated human blood cells (RBCs and platelets), nucleated human blood cells (lymphocytes) and nucleated fowl blood cell nuclei were analyzed. The stained blood cells were incubated at three different incubation temperatures (20, 25 and 37ºC) and observed under fluorescent microscope. When the sample of human RBCs and platelets was stained with diluted C-PC, fluorescence was not seen, indicating negative results (Table 3). In the second set of experiments, human lymphocytes and fowl blood cell nuclei were tested and it was observed that this dye reacted very strongly, showing a titer of reactivity $>10^{-6}$ (Table 3). The reactivity was directed towards the cell nuclei rather than the cell surface, indicating that the dye penetrates the cell and stained the nuclei (Figure 3). Fluorescent was not observed for cytoplasm or cell wall.

Binding affinity of C-PC to Genomic DNA

The binding affinities of C-PC was analyzed with human and plant genomic DNA at different incubation temperatures and dilution ratios. The maximum binding affinity of C-PC observed in both human and plant genomic DNA at 25°C. It was also amazing to observe that C-PC dilution had no effect on the sharpness of the genomic DNA at constant DNA concentration. The C-PC stained DNA even at a high dilution (10^{-6}) performed same results as with purified C-PC. The genomic DNA could thus be stained and detected by the C-PC. The

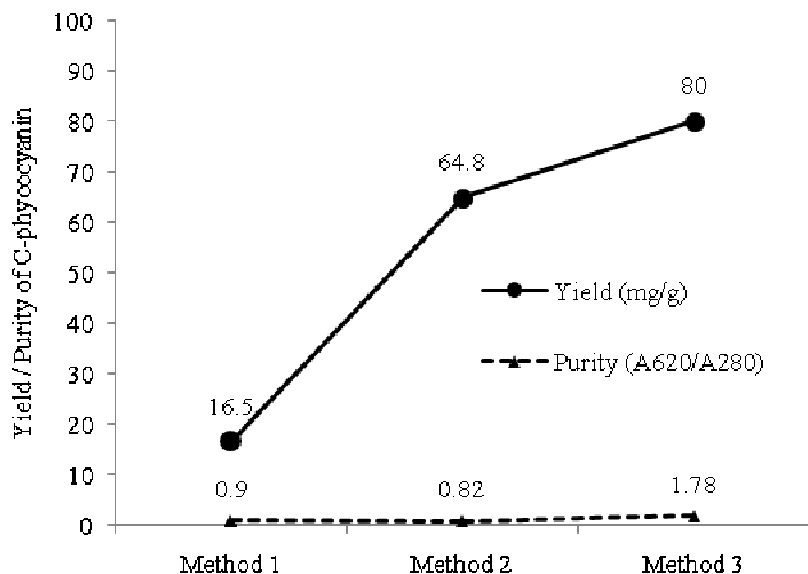

Figure 2: Correlation coefficient between the variables of yield and purity of C-PC by different drying methods of the algal biomass (Method 1: Drying in water bath, Method 2: Drying in direct sun light, Method 3: Air-drying)

Table 3: The binding affinity of C-PC to various cells and nuclei

Cell types stained with C-PC	No. of samples	Incubation temp. (ºC)			Binding affinity at dilution
		4	25	37	
Human RBCs	5	-	-	-	Nil
Human platelets	5	-	-	-	Nil
Human Lymphocytes	5	-	-	-	Nil
Fowl RBC Nuclei	5	-	++	+	10^{-6}

The +ve sign indicates positive results (fluorescence) and –ve sign indicates negative results (no fluorescence).

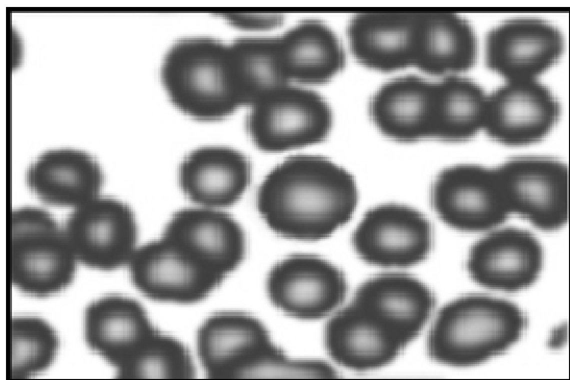

Figure 3: Nucleated cells of human lymphocytes stained with C-PC (magnification 40x)

fluorescent image of stained human and plant genomic DNA is shown in Figure 4.

Staining ability of C-PC in comparison to ethidium bromide

A comparative study was carried out on the binding affinities of C-PC and ethidium bromide with human and plant genomic DNA. The binding affinity of C-PC was observed in both human and plant genomic DNA. The DNA bands resulted from C-PC staining were more or less similar than ethidium bromide staining as shown in Figure 5.

DISCUSSION

Phycobiliproteins such as phycoerythrin and allophycocyanin have been extensively used in staining

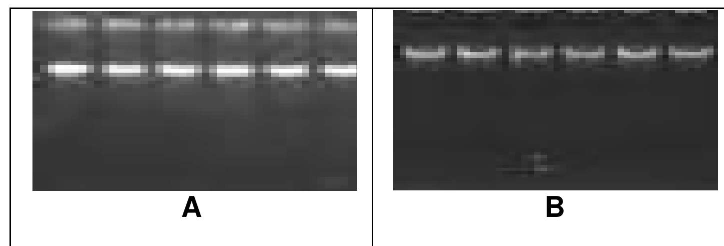

Figure 4: Human (A) and plant (B) genomic DNA stained with C-PC

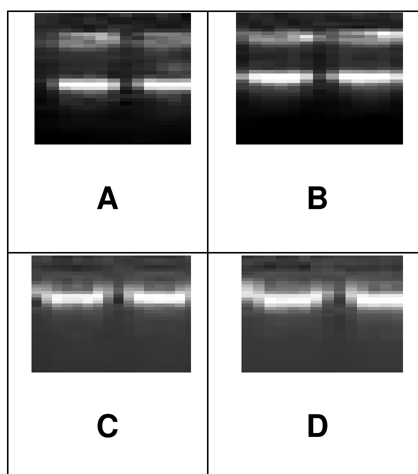

Figure 5: Staining ability of ethidium bromide and C-PC. (A) Human genomic DNA stained with ethidium bromide, (B) Human genomic DNA stained with C-PC, (C) Plant genomic DNA stained with ethidium bromide, (D) Plant genomic DNA stained with C-PC

of DNA and in diagnostic studies fluorescent marker in the fluorescent immunoassay (Kulkarni et al., 1996; Kuddus and Ramteke, 2009), similarly we investigated the dye, C-PC, for its potential use in the immunodiagnostic field and as a non-toxic natural DNA stain. From the present study, it can be concluded that the purified C-PC has great affinity towards the nucleus. Even the morphology of the nucleus can be clearly viewed and may be used to count nucleated cells. Harding and coworkers (Harding, 2000) visualized apoptotic cells, labeled with a streptavidin-allophycocyanin conjugate.

The utilization of different stain/dye agents has greatly assisted the study of complex biological interactions in the field of biology. In particular, fluorescent labeling of biomolecules has been demonstrated as an indispensable tool in many biological studies. Fluorescence microscopy is an influential tool in life science research, and recent years have witnessed a rapid development of technique and application, from FRET, BRET and FLIM to TIRFM and other live cell imaging approaches (Kuddus and Ramteke, 2009). Purified C-PC has nutraceutical and pharmaceutical potentials (Belay, 2002). A variety of impaired physiological conditions are reported to be relieved by C-PC administration (Farooq *et al*., 2004; Chiu *et al*., 2006; Kahn *et al*., 2006; Riss *et al*., 2007; Sathyasaikumar *et al*., 2007). It has also been observed that C-PC can inhibit cell proliferation (Liu *et al*., 2000), induce apoptosis in cancerogenic cell lines (Subhashini *et al*., 2004; Roy *et al*., 2007), inhibit the other enzyme activities and affect gene regulation in mammalian cell lines (Madhyastha *et al*., 2006; Cherng *et al*., 2007). The deep blue colour of phycocyanin has been used as a naturally occurring colorant for food additive purposes (Kato, 1994; Hirata *et al*., 2000). A few studies have addressed the functionality of C-PC in foods with regards to colour stability (Jespersen *et al*., 2 005; Mishra *et al*., 2008) and

rheological properties (Batista *et al*., 2006). The positive health effects have in most cases been attributed to antioxidant and radical scavenging activities of C-PC. The antioxidant and radical scavenging activities of C-PC from different cyanobacteria are well documented (Bhat and Madyastha, 2000; Dasgupta *et al*., 2001; Upasani and Balaraman, 2003; Benedetti *et al*., 2004; Bermejo *et al.,* 2008; Soni *et al*., 2008). C-PC will have wide applications in fluorescence microscopy and in the diagnosis and prognosis of diseases such as chronic lymphatic leukemia. C-PC conjugates are useful in multi-color flow cytometry with instruments equipped with a laser that will excite the C-PC within its absorbance range. It may be conjugated to monoclonal and polyclonal antibodies for use in multicolor FACS analysis. The C-PC can be applied in counting of nucleated cells and measuring the proliferative activity of the cell in case of malignancy.

This study also highlights affinity of C-PC towards genomic DNA. The dye migrates very well on gel electrophoresis without dissociation even at 10^{-6} dilutions. Also, because the dye is natural in origin and has been found to be without any toxic effects (Arad and Yaron, 1992), unlike the conventional synthetic fluorochromes, it can act as a substitute for the generally used ethidium bromide, which is a carcinogenic compound. Ethidium bromide is much cheaper than the APC but due to its carcinogenic nature it may be substituted by natural stain such as C-PC. C-PC has no specificity for any other proteins because it cannot stain them so it can be suitably employed as a specific stain for DNA staining and in genomic DNA analysis, and as a marker in the immuno-fluorescent techniques. By using this natural dye as a marker various blood cells/molecules may be detected by adapting avidin/biotin as a non-conventional coupling agent. C-PC from *A. platensis* in combination with streptavidin used as fluorescent probes in cytometry (Telford *et al*., 2001). C-PC also used as fluorescent probe for online monitoring of growth in cyanobacterial cultures (Sode *et al*., 1991), detection of toxic cyanobacteria in drinking water (Izydorczyk *et al*., 2005) and remote sensing of cyanobacteria in natural water bodies (Simis *et al*., 2005). We think it likely that C-PC can be conjugated with equal facility to toxins, hormones, growth factors and other biologically interesting molecules for application to a wide range of highly sensitive fluorescence analysis.

Conclusion

C-PC pigment could be extracted from algal biomass following the drying method under shadow at 25°C by air circulation at a large scale. The partially purified C-PC can be suitably diluted to an appropriate concentration to stain different cells, tissues and genomic DNA. The commonly used synthetic dyes including ethidium bromide could be replaced by the naturally available C-PC pigments for the detection of genomic DNA. The C-PC pigment without any toxic effect has applications in the several industries including food, cosmetics, clinical research or immunological laboratories as labels for antibodies, receptors and for the diagnosis of biological molecules. This natural nuclear stain extracted from *Spirulina* is to be utilized for the preparation of stains at appropriate concentrations and sold commercially.

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