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Full Length Research Paper

Binding affinity of allophycocyanin to blood cells and nuclei

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Allophycocyanin (APC) was extracted from *Anacystis nidulans* and examined for its binding affinity towards various cells/nuclei. The various cells and nuclei isolated from human, rat and rabbit blood were stained with APC and examined under fluorescent microscope. Allophycocyanin dye showed high affinity towards nucleus rather than the cell surface. It reacts with the human lymphocytes at high dilution of greater than 10^{-6}. The APC was seen to have no affinity towards anucleated cells such as RBCs and platelets from human, rat and rabbit. Thus, APC may be used to count nucleated cells and can also be employed as potential diagnostic agent to differentiate nucleated cell from anucleated cells. The study also highlights affinity of APC towards genomic DNA and it has no specificity for any other proteins because it could not stain them and thus could be suitably employed in genomic DNA analysis and may be replaced by harmful chemicals used for staining.

Key words: Allophycocyanin, DNA staining, fluorescent microscopy, *Anacystis nidulans*.

INTRODUCTION

Allophycocyanin (APC), a protein that belongs to the light-harvesting phycobiliprotein family, is emerging as one of the brightest fluorescent probes. The phycobiliproteins are water-soluble fluorescent proteins derived from cyanobacteria and eukaryotic algae. In these organisms, they are used as accessory or antenna pigments for photosynthetic light collection. They absorb energy in portion of the visible spectrum that is poorly utilized by chlorophyll.

Phycobiliproteins contain multiple chromophore prosthetic groups, which are responsible for the fluorescent properties of these proteins. They have high quantum yields that are constant over a broad pH range.

The isoelectric point of phycobiliproteins ranges from 4.7 to 5.3 (Glazer, 1981). Oi and coworkers introduced the phycobiliproteins as a novel class of fluorescent tags in 1982 (Oi et al., 1982). These naturally occurring fluorescent proteins immediately became of wide spread use in many diagnostic and clinical assay, in histochemistry and in diverse research applications (Glazer and Stryer, 1990).

APC is a turquoise-blue coloured protein of molecular weight ranging from 80 to 110 kDa, containing six chromophore prosthetic groups, which possesses α and β subunits with an apparent $(\alpha\beta)_3$ quaternary structure. The absorption and emission maxima of APC are 650 and 660 nm, respectively (Trinquet et al., 2001). To the best of our knowledge, no work has been carried out on staining of blood cells/nuclei by APC. The majority of stains used in hematology and in staining of various cells are of the synthetsic type that may cause biohazards. Hence, an attempt was made to study the property of APC for staining of blood cells/nuclei and genomic DNA. The APC was extracted from *Anacystis nidulans* and pu-

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Abbreviations: APC, Allophycocyanin; **EDTA**, ethylene diamine tetra-acetic acid; **PBS**, phosphate buffer saline; **FRET**, fluorescence resonance energy transfer; **BRET**, bioluminescence resonance energy transfer; **FLIM**, fluorescence lifetime imaging microscopy; **TIRFM**, total internal reflection fluorescence microscopy; **FACS**, fluorescence activated cell sorter; **RBCs;** red blood cells.

rified by chromatographic techniques and used in all the experiments reported in this paper.

EXPERIMENTAL PROCEDURE

Isolation of RBCs from blood of humans, rats, and rabbits

RBCs were separated from freshly collected blood samples from humans, rats, and rabbits. Blood samples from healthy animals were collected in EDTA-coated collection tubes aseptically. The RBCs were packed by centrifugation in cold condition. The cells were separated from plasma, washed with isotonic saline and then resuspended in same saline solution (Bhatia, 1977).

Isolation of human platelets

The human platelets were isolated by differential centrifugation. The blood samples collected from human were initially centrifuged at 800 rpm at room temperature. The resulting platelets rich plasma was transferred in another centrifuge tube and centrifuged at 2000 rpm at room temperature. A platelet button was formed. The harvested platelets were washed with 0.01 mM phosphate buffer saline (pH 7.2) and resuspended in the same buffer (Bhatia, 1977).

Isolation of human lymphocytes

The human lymphocytes from freshly collected blood were separated using Ficoll-Hypaque (Histopaque-1077, Sigma) density gradient centrifugation. The blood was diluted in the ratio of 1:2 with normal saline. In a centrifuge tube, 6 ml of Ficoll-Hypaque (density – 1.077 g/ml) was added and 3 ml of blood sample was layered upon it carefully. Centrifugation was carried out at 400 × g for 30 min. The mononuclear cells were recovered at the Ficoll-Hypaque interface in the form of white band. The cells at the interface were aspirated with a Pasteur pipette. Washing of the cells was done in large volume of saline at a speed of 2500 rpm. The pellet was suspended in 0.01 mM PBS, pH 7.2 with 5% bovine serum albumin (BSA) (Yadava and Mukherjee, 1992). The cells were treated with 0.3% saponin for permeabilization and washed with buffer before staining.

Extraction of genomic DNA

Fresh human blood was collected and DNA was isolated by using DNA extraction kit (KT-23, Bangalore Genei, India) as per manufacturer's protocol. After isolation of genomic DNA, concentration of total DNA was calculated from the absorbance at 260 nm (an A_{260} of 1 corresponds to 50 µg/ml ds DNA solution) and then it is diluted to maintain the concentration 100 µg/ml. Purity of the DNA was checked by ratio of absorbance at 260 and 280 nm.

Dilution of purified allophycocyanin

The allophycocyanin was isolated from *A. nidulans* by modified method of Trinquet et al. (2001) and purified by ammonium sulphate precipitation and chromatographic techniques (unpublished). Protein concentration in mg/ml was determined using the specific extinction coefficient of 6.35 at 650 nm, pH 7.0 (Brown and Troxler, 1977). The absorbance and fluorescence excitation and emission spectra of the purified APC was identical to those previously shown for APC. The purified APC was stored in the dark at 4°C. The purified allophycocyanin (1 mg/ml) was serially diluted up to 10^{-6} with 0.01 mM PBS, pH 7.2 and each dilution was used in all the experiments reported here.

Staining of cells/nuclei with allophycocyanin

To serially diluted APC, 5% of RBCs from human, rat and rabbit, 5% of human platelets and 10% of human lymphocytes were added. The cells were incubated for 30 min in the dark at different temperatures (4, 25 and 37°C). The cells were then washed with their respective media. A drop of this was mounted on the slide (chambered cover slips, Sigma) with mounting medium (glycerol and distilled water, 9:1). The fluorescence was visualized under an Olympus fluorescent light microscope (CX31) with fluorescence attachment (CX-RFL-2) containing blue and green filter cube for excitation. The microscope was connected with an Olympus digital camera and figures were made from digitized image. APC dye is exited at 650 nm and emits fluorescence at 660 nm.

Staining of genomic DNA

The isolated DNA was analyzed by gel electrophoresis. The DNA was stained and detected by purified and diluted allophycocyanin instead of the generally used fluorescent dye ethidium bromide. Along with the test sample the standard DNA molecular weight marker (Bangalore Genei, India) was run as positive control and different proteins like bovine serum albumin and papain (10 µl) were also run as negative controls. For gel electrophoresis 0.8% agarose and Tris-acetate-EDTA (TAE) buffer was used. 10 µl of genomic DNA (100 µg/ml), 10 µl of standard DNA molecular weight marker (100 µg/ml) and 10 µl of BSA and papain dissolved in TAE buffer, were incubated with 50 µl of diluted allophycocyanin dye in an eppendorf tube for 10 min at 25 ± 2°C. This was loaded in the wells with the loading buffer (0.25% Bromophenol blue, 0.25% xylene cyanol and 40% sucrose in distilled water). Electrophoresis was carried out at 50 volts. After electrophoresis, the gel was observed for the presence of DNA stained with APC on Vilber Lourmate's trans-illuminator (France) and photographed with a Gel Documentation system (GeneLine, Spectronics Corporation, New York).

RESULTS AND DISCUSSION

The purified allophycocyanin from *A. nidulans* was tested with various batches of human, rat and rabbit RBCs, human lymphocytes, and platelets to determine its binding affinity either to the cell surface or cell nuclei. When the sample of human, rat and rabbit RBCs and human platelets was stained with purified and diluted allophyco-cyanin, fluorescence was not seen under fluorescent microscope, indicating negative results (Table 1).

In the second set of experiments, human lymphocytes obtained from different donors were tested with allophycocyanin and it was observed that this dye reacted very strongly, showing a titer of reactivity $>10^{-6}$ (Table 1). 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 1). Fluorescent was not observed for cytoplasm or cell wall.

The allophycocyanin was further tested for its binding affinity towards the genomic DNA by employing electrophoretic technique. Five batches of genomic DNA samples were tested. With each of the batches, a stan-dard DNA molecular weight marker and negative controls were also run simultaneously. The genomic DNA sample and DNA marker were seen to fluoresce, but no fluores-

Table 1. Binding affinity of purified allophycocyanin to various cells.

Cells used	No. of Samples	Incubation Temp. (ºC)			Binding affinity at dilution
		4	25	37	
Human RBCs	8	-	-	-	Nil
Rabbit RBCs	5	-	-	-	Nil
Rat RBCs	5	-	-	-	Nil
Human Platelets	5	-	-	-	Nil
Human Lymphocytes	5	N	+	N	10^{-6}

+ = Positive results (fluorescence); - = negative results (no fluorescence); N = not done.

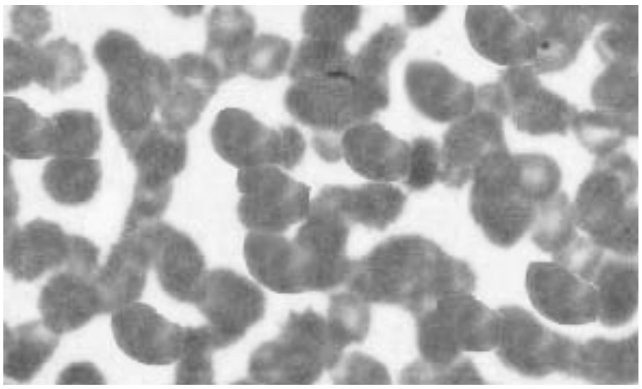

Figure 1. Fluorescent staining of lymphocytes with purified APC from *Anacystis nidulans* (magnification x1,000).

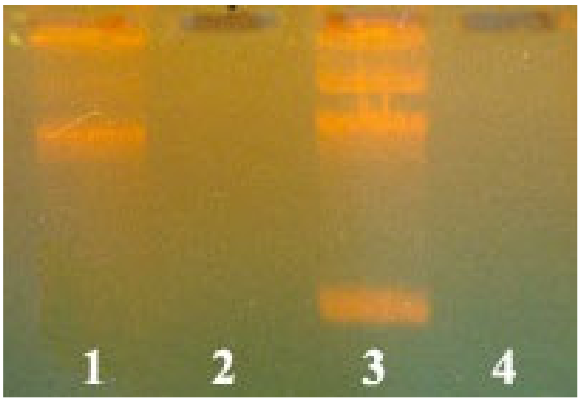

Figure 2. Fluorescent staining of DNA and different proteins with purified APC. Lane 1: Human genomic DNA; Lane 2: BSA ; Lane 3: standard DNA marker ; Lane 4: papain.

scence was seen along the path of the other proteins (Figure 2). It was also amazing to observe that APC dilution had no effect on the sharpness of the human genomic DNA at constant DNA concentration. The APC-stained DNA even at a high dilution (that is, 10^{-6}) performed same results as with ethidium bromide. The genomic DNA could thus be stained and detected by the allophycocyanin.

Phycobiliproteins such as phycoerythrin and phyco-cyanin have been extensively used in staining of DNA and in diagnostic studies; for instance, biotinylated phycocyanin was employed as a fluorescent marker in fluorescent immunoassay to detect the Rh antibody coated on the RBC surface (Kulkarni et al., 1996). We similarly investigated the dye (APC) for its potential use in the immunodiagnostic field and as a DNA stain. From the present study, it can be concluded that the purified allophycocyanin has a great affinity towards the nucleus. Even the morphology of the nucleus can be clearly viewed and may be used to count nucleated cells. Harding et al. (2000) visualized apoptotic cells, labeled with a streptavidin-allophcocyanin 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 the technique has, in recent years, undergone a rapid development and application, from FRET, BRET and FLIM to TIRFM and other live cell imaging approaches. Batard and coworkers (2002) used APC as a donor in FRET-based time resolved fluorescence sensing. It was also applied in FACS analysis by Sabine et al. (2002). Ge and coworkers (2005) purified recombinant allophyco-cyanin expressed in *Escherichia coli*. The antioxidant and anti-tumour activities of recombinant allophycocyanin was concluded by Ge et al. (2002). Allophycocyanin will have wide applications in fluorescence microscopy and in the diagnosis and prognosis of diseases such as chronic lymphatic leukemia. Allophycocyanin conjugates are useful in multi-color flow cytometry with instruments equipped with a laser that will excite the APC within its absorbance range. It may be conjugated to monoclonal and polyclonal antibodies for use in multicolor FACS analysis. The allophycocyanin can be applied in counting nucleated cells and measuring the proliferative activity of the cell in case of malignancy.

The study also highlights the affinity of APC for 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 carcinogenic. Ethidium bromide is much cheaper than the APC but due to its carcinogenic nature, it may be substituted by natural stains such as APC. Allophycocyanin has no specificity for other proteins because it could not stain them; so it could 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. Schmid et al. (2000) used APC for cell surface staining in combination with Alexa488, a green fluorescent fluorochrome. We think it likely that APC 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.

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