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C-Phycocyanin from *Oscillatoria tenuis* exhibited an antioxidant and *in vitro* antiproliferative activity through induction of apoptosis and G_0/G_1 cell cycle arrest

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ABSTRACT

This study was undertaken to develop an efficient single step chromatographic method for purification of *C*-phycocyanin (CPC) from species of *Oscillatoria tenuis*. Purification of CPC involves a multistep treatment of the crude extract by precipitation with ammonium sulphate, followed by gel filtration chromatography. Pure CPC was finally obtained from *O. tenuis* with purity ratio (A_{620}/A_{280}) 4.88. SDS–PAGE of pure CPC yielded two bands corresponding to α and β subunits; the molecular weight of α subunit is 17.0 kDa, whereas the molecular weight of β subunit is 19.5 kDa. Fluorescence and phase contrast microscopy revealed characteristic apoptotic features like cell shrinkage, membrane blebbing, nuclear condensation and DNA fragmentation. CPC exhibited antioxidant and antiproliferative activity against human cancer cells through apoptosis; nuclear apoptosis induction was accompanied by G_0/G_1 phase arrest and DNA fragmentation. CPC is a natural pigment with potential as an anticancer agent.

1. Introduction

Chemoprevention is an effective way to reduce cancer risk. Natural products have been the mainstay of cancer chemotherapy for the past 30 years. Blue-green algae are the most primitive life forms on earth with nutrient-dense, edible forms like *Nostoc*, *Spirulina*, *Aphanizomenon* species, etc. The cyanobacterial phycocyanin (CPC) is the major phycobiliprotein in cyanobacteria. This blue colour red fluorescing biliprotein was first reported in 1928 by Lemberg (Patil et al., 2006). It consist of a protein component and a chromophore, and the protein moiety consists of α and β subunit (Patil et al., 2006). Phycocyanin is a natural blue colourant, has uses as a food colourant for chewing gum, sorbets, soft drinks, candies and cosmetics, including lipstick and eyeliners. Small quantities are also used as biochemical tracers in immunoassays due to its fluorescent properties (Silveira, Burket, Costa, Burket, & Kalil, 2007).

Phycocyanin has been proven to have therapeutic properties including antioxidant, anti-inflammatory and anti-cancer activities (Eriksen, 2008; Madhyastha, Sivashankari, & Vatsala, 2009). Recent studies have demonstrated antioxidant, anti-mutagenic, antiviral, anticancer, anti-allergic, immune enhancing, hepatoprotective, blood vessel-relaxing and blood lipid-lowering effects of *Spirulina* extracts. The biological and pharmacological properties of *Spirulina* were attributed mainly to calcium-spirulan and CPC (Subhashini et al., 2004). The cost of phycocyanin products varies widely and is dependent on the purity ratio, which is defined as the ratio of absorbance at 620 and 280 nm (A_{620}/A_{280}).

CPC has been purified using a number of combinations of chromatographic steps such as ion exchange chromatography, gel filtration chromatography, chromatography on hydroxyapatite and expanded bed adsorption chromatography (Bermejo et al., 2003; Santiago-Santos, Ponce-Noyola, Olvera-Ramırez, Ortega-Lopez, & Canizares-Villanueva, 2004; Soni, Kalawadia, Trivedi, & Madamwar, 2006; Wang, 2002). In most cases, it has been purified by sequential application of either cation or anion exchange chromatography, followed by size exclusion chromatography as a final polishing step. Though purification was achieved in the above methods, high purity methods were very expensive while low-cost

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methods were time-consuming. Moreover, the combination of several steps decreases the yield considerably, requiring large culture volumes for purification (Jobby & Sharma, 2003). Hence the present study attempted to simplify the purification process as well as reduce the cost of process. Additionally we were interested to study the biological activities of CPC from *Oscillatoria tenuis*.

2. Material and methods

2.1. Chemicals and reagents

Seralose 6B gel filtration column, Griess reagent and ascorbic acid were purchased from SRL Chemicals, Mumbai, India. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and Dulbecco's Modified Eagles Medium (DMEM) were purchased from Hi Media, Mumbai, India. Nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide (NADH), (4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid) (HEPES), penicillin, streptomycin, propidium iodide, ethylenediaminetetraacetic acid (EDTA), WST-8 kit, and dimethyl sulphoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO). Foetal bovine serum was purchased from Gibco. LDH assay kit was purchased from Cayman Chemicals (Ann Arbor, MI). All other chemicals, including solvents, were of high purity and of analytical grade.

2.2. Organism and culture condition

The freshwater cyanobacterium *O. tenuis* was obtained from Algal culture collection, CAS in Botany, University of Madras, India. These organisms were grown in batch cultures in BG 11 medium (Rippka, Deruelles, Waterbury, Herdman, & Stanier, 1979) at pH 7.5 and 20 ± 2 °C with optimum light intensity of 60 μE m^{-2} s^{-1} provided by cool-white fluorescent tubes with a dark:light cycle of 12:12 h.

2.3. Phycobiliprotein extraction

All buffers and reagents used in purification were prepared in Milli Q water and were added with 0.01% sodium azide. 20 L of 30th days old algal culture were recovered by centrifugation at 4000*g*, at 4 °C for 20 min. Cell pellets were washed with 0.02 M sodium phosphate (pH 7.2). The washed cell mass was crushed in mortar and pestle by addition of liquid nitrogen. Twenty-five grams of finely powdered frozen cell mass was obtained and thawed by addition of 100 mL of the same buffer. The resultant slurry was centrifuged at 10,000*g* for 20 min to remove the cell debris and blue supernatant-crude cell free extract was used for the following purification steps.

2.4. Phycocyanin purification

2.4.1. Ammonium sulphate precipitation

The whole procedure was carried out at 4 °C. Finely powdered $(NH_4)_2SO_4$ was gradually added to the crude extract to achieve 20% saturation with continuous stirring for 2 h. The resulting solution was kept under static conditions for 4 h and centrifuged at 17,000*g* for 20 min. The supernatant was pooled and subjected to 55% $(NH_4)_2SO_4$ saturation in a manner similar to that of 20% saturation. After the static incubation, the solution was centrifuged at 17,000*g* for 20 min. The pellet was re-suspended in 15 mL of 0.02 M sodium phosphate buffer (pH 7.2) dialysed with 0.01 M sodium phosphate buffer (pH 7.2) and lyophilised for further study.

2.4.2. Purification of CPC by gel filtration chromatography

The crude CPC dissolved in 0.01 M sodium phosphate buffer (pH 7.2) was applied to a Seralose 6B gel filtration column (3 × 100 cm and 320 mL bed volume), followed by elution with 0.01 M sodium phosphate buffer at a flow rate of 30 mL/h. Eluant (5 mL/tube) was collected and blue colour fraction was measured to determine CPC content and purity. Finally, high purity fractions were pooled together and lyophilised for further study. The protein estimation was carried out with previously described method of Lowry et al. (1951).

2.4.3. Spectrophotometric estimation of phycobiliproteins

UV–vis absorbance spectra were recorded on double-beam 1800 UV–vis spectrophotometer (Shimadzu, Kyoto, Japan). The amount of CPC, CPE (*C*-phycoerythrin), and CAPC (*C*-allophycocyanin) in the sample was calculated using the simultaneous equations described in Soni et al. (2006), as follows:

$$\text{CPC (mg/mL)} = (OD_{620} - 0.7\ OD_{650})/7.38 \quad (1)$$

$$\text{Recovery (\%)} = 100 \times (\text{CPC (mg/mL)} \times \text{collected volume (mL)}) / (\text{CPC initial extract (mg/mL)} \times \text{initial extract (mg/mL)}) \quad (2)$$

2.4.4. FTIR

A Perkin Elmer Spectrum RX I FTIR spectrophotometer (Waltham, MA) was used in the measurements that were all performed in a dry atmosphere at room temperature (23 ± 0.5 °C). The different stages (Stage-I, Stage-II and Stage-III) of CPC were collected and it was then compressed into a thin KBr disc under a pressure of 7845 kPa for 5 min. The functional groups present in the *O. tenuis*, in different stage purification of CPC were determined by the FT-IR studies. Regardless of the sample preparation procedure in this study, all spectra were recorded within a range of 4000–400 cm^{-1}. Spectral treatment consisted of background subtraction, baseline correction and normalisation. The statistical package ORIGIN 6.0 Microcal 1999 (Microcal Software, Inc., Northampton, MA) was employed for the chemometric calculations.

2.4.5. Native and SDS–PAGE

Native PAGE was carried out on an 8% (w/v) polyacrylamide slab gel, 1.5 mm thick using Tris–glycine buffer (pH 8.3) and the bands were visualised by staining with silver staining. SDS–PAGE was carried out using a 15% (w/v) polyacrylamide slab gel, 1.5 mm thick, containing 0.1% (w/v) SDS with a stacking gel of 4% acrylamide and 0.1% bis-acrylamide. Samples were pre-incubated with 2% (w/v) SDS, 10% (v/v) glycerol, 4.5% (v/v) β-mercaptoethanol, 0.025% (w/v) bromophenol blue and 60 mM Tris (pH 6.8), for about 5–10 min at 95 °C. Gels were run at room temperature and visualised by staining with silver staining. The molecular weights of subunits were determined by calibrating the gel with low molecular weight markers (Sigma).

2.4.6. Antioxidant properties of purified CPC

2.4.6.1. Free radical-scavenging activity. Anti-oxidant activity was measured for the purified CPC, based on scavenging of the stable free radical, DPPH, using a previously described method (Lim et al., 2007). The purified CPC in PBS was added to 180 μL of a solution that contained DPPH. After 30 min, the absorbance was measured at a wavelength of 492 nm, using an enzyme-linked immuno-sorbent assay (ELISA) plate reader (Thermo Multiskan, Waltham, MA). The free radical-scavenging activity was expressed as follows:

$$\text{Scavenging \%} = [1 - A_{\text{sample}} - (A_{\text{blank}}/A_{\text{control}})] \times 100$$

L-Ascorbic acid was used as a positive control.

2.4.6.2. Hydroxyl radical-scavenging activity assay. The scavenging activity of CPC against the hydroxyl radical was investigated using Fenton's reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$) and method