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Isolation, characterization and antioxidative activity of C-phycocyanin from *Limnothrix* sp. strain 37-2-1

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ABSTRACT

C-phycocyanin (C-PC) is a blue colored accessory photosynthetic pigment found in cyanobacteria. Some of the medicinal properties of *Spirulina* have been attributed to this pigment, which includes anticancer, antioxidant, and anti-inflammatory activity. We have screened cyanobacteria isolated from freshwater habitats in Florida for their high content of C-PC. Of 125 strains tested, one filamentous strain identified as *Limnothrix* sp. was selected for further research. This strain produced 18% C-PC of total dry biomass. Here we describe a simple method for obtaining C-PC of high purity without the use of ion exchange chromatography. The procedure is based on pigment precipitation from the cell lysate with an appropriate concentration of ammonium sulfate, then purification with activated carbon and chitosan, followed by a sample concentration using tangential flow filtration. We have shown that when the lower concentration of ammonium sulfate was used, C-PC with higher purity index was recovered. Characterization of C-PC from *Limnothrix* showed that it had an absorbance maximum at 620 nm and fluorescence at 639 nm. The molecular mass of intact C-PC was estimated to be ~50 kDa with α and β subunits forming dimmers. When C-PC content per unit biomass was compared to that of marketed *Spirulina* powder, we found that *Limnothrix* was superior. C-phycocyanin from *Limnothrix* had an antioxidative activity on DPPH free radicals similar to that found in a natural antioxidant – rutin.

1. Introduction

C-phycocyanin is a blue colored photosynthetic accessory pigment that absorbs light at about 620 nm and emits fluorescence at about 640 nm. It is a phycobiliprotein with a molecular mass of about 110 kDa containing two subunits (Glazer and Stryer, 1984). Unlike other phycobiliproteins (phycoerythrin and allophycocyanin), C-PC is a major accessory pigment and it is present in all cyanobacteria. This pigment is traditionally isolated from *Spirulina* (Boussiba and Richmond, 1979; Herrera et al., 1989; Patil et al., 2006; Bhaskar et al., 2005) however, some other cyanobacterial genera have also been used (Santiago-Santos et al., 2004).

Isolation and purification of C-PC is a multistep process that includes fractional precipitation with ammonium sulfate, ion exchange chromatography and gel filtration (Herrera et al., 1989; Zhang and Chen, 1999; Bhaskar et al., 2005). Besides this general procedure, attempts were made to optimize C-PC purification by including the use of rivanol (Minkova et al., 2003), chitosan and charcoal (Patil et al., 2006), and hydrophobic interaction chromatography (Soni et al., 2008).

Spirulina has been reported to have a number of medicinal properties (Belay et al., 1993; Gantar and Svircev, 2008), some of which have been attributed to C-PC. One of the first reports on beneficial effect of C-PC (Belay et al., 1993) cites the Japanese patent #58-65216 (Dainippon Ink & Chemicals, 1983), which claims that this blue pigment from *Spirulina* significantly increases the survival rates of mice that had been injected with liver tumour cells. It has been further suggested that stimulation of the immune system by C-PC was a mechanism that inhibited growth of tumour cells. In fact, a number of authors have described the induction of apoptosis as a mechanism of C-PC activity (Reddy et al., 2003; Bobbili et al., 2003).

C-PC was shown to suppress allergic inflammation reactions in different animal models. This pigment reduces inflammation of the small intestine in mice by suppressing the level of antigen specific IgE antibody (Nemoto-Kawamura et al., 2004), prevents colonic damage in acetic acid-induced colitis in rats (Gonzales et al., 1999), and inhibits the induced mouse ear oedema by reducing PGE2 (prostaglandin E2 levels) (Romay et al., 1998). Remirez et al. (2002) suggest that the inhibition of allergic inflammatory response by

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C-PC is mediated, at least in part, by inhibition of histamine release from mast cells. Hypocholesterolemic effect of *Spirulina platensis* concentrate was attributed to C-PC and its effect on inhibition of both jejunal cholesterol absorption and ileal bile acid reabsorption (Nagaoka et al., 2005).

Besides a potential use for therapeutic purposes, C-PC is being used as a fluorescent marker in biomedical research (Glazer, 1994). Apparently, there is a great interest for C-PC research and therefore productive organisms and new technologies for its isolation and purification are needed. In this work, we assess the ability of different cyanobacterial strains to produce C-PC in amounts equal or higher to those obtained from traditionally used *Spirulina*. We also describe a new and simple technique for isolation of this pigment. In addition, we have characterized the pigment obtained from the novel strain of *Limnothrix* and assessed its antioxidative activity.

2. Materials and methods

2.1. Screening of organisms

We screened 125 cyanobacteria collected from freshwater habitats in Florida from our culture collection for the isolates with high C-PC content. Taxonomic identity of the isolates was based on morphological characteristics (Anagnostidis and Komarek, 1988). The taxonomic identity of the strain that showed the highest content of C-PC, was confirmed by 16S rRNA gene sequencing and BLASTN search. Isolation of the total genomic DNA, 16S rRNA gene amplification, and its sequencing were performed as described elsewhere (Myers et al., 2007). The cyanobacteria specific primers CYA359F and CYA781R(b) (Nübel et al., 1997) were used for PCR amplification and the sequencing of the 16S rRNA gene.

To determine the C-PC content in the individual strains, all strains were grown in 3-liter cultures in BG11 medium (Rippka et al., 1979) under cool-white light (50 $\mu E\, m^{-1}\, s^{-1}$), temperature of 25 °C, and aeration with sterile air. The biomass was harvested by centrifugation, freeze dried, and kept in a freezer until further use. In order to obtain the cell extract, 100 mg of dry biomass was suspended in 10 ml of 0.1 M phosphate buffer pH 7.0 and left in refrigerator (4 °C) for overnight extraction. Those cell extracts that showed blue color, were centrifuged and filtered through 0.45 μm pore size filters (Millipore, Billerica, MA) and the amount of C-PC was determined spectrophotometrically (Bennett and Bogorad, 1973).

2.2. Isolation and purification of phycocyanin

For isolating larger amounts of C-PC and for the purpose of optimizing the purification process, the selected strain *Limnotrhrix* sp. 37-2-1 was grown in a 30-liter bioreactor under the same condition as described above. The harvested biomass was suspended in distilled water (Silveira et al., 2007) and repeatedly freeze–thawed (Soni et al., 2006; Zhang and Chen, 1999) at least three times for the purpose of lyzing the cells. The crude cell lysate, which had an intense blue color, was sequentially filtered through Whatman 43, Whatman GF/B (Maidstone, UK), and 0.47 μm Millipore filters (Billerica, MA).

This crude extract was stirred on a magnetic stirrer with activated carbon (1%, w/v) (Darco, 20-40 mesh granulated, Sigma, St. Louis, MO, USA) and chitosan (Sigma, St. Louis, MO, USA) to a final concentration of 0.01 $g\, l^{-1}$. After 15 min of stirring, the crude extract was centrifuged at 4000 × *g* and the supernatant was precipitated with different concentrations of ammonium sulfate, ranging from 20% to 60% (with increments of 5%). The precipitation was done overnight at 4 °C. The precipitate was separated by centrifugation at 14,000 × *g* and temperature of 15 °C. The precipitate was re-suspended in 0.1 M phosphate buffer pH 7.0. Desalting and concentrating the pigment was performed by a tangential flow filtration system (Labscale TFF System, Millipore, Billerica, MA, USA) using a membrane with the pore size of 30 kDa (Pellicon XL, PLCTK 30, Millipore, Billerica, MA, USA). The purity of the pigment was assessed by calculating the ratio of absorbencies at 620/280 (Boussiba and Richmond, 1979) and with using SDS-PAGE, which was supposed to reveal the presence of contaminating proteins. Pigment concentration and its purity (620/280 ratio) was determined in each individual step during the purification process (Table 2). The purified phycocyanin was freeze-dried and kept in a refrigerator until further use. The presented data are the means of measurements from three different experiments by using the same batch of cyanobacterial biomass.

For comparisons reasons, the phycocyanin was also isolated from powdered *Spirulina* samples manufactured and marketed by two different companies (designated as *Spirulina* #1 and *Spirulina* #2) by using the same procedure. For this experiment, the *Limnothrix* biomass was freeze dried and used for extraction. The *Spirulina* samples were purchased in health food stores.

2.3. Spectroscopic measurements

During the purification process, the absorption of C-PC in the visible and UV range was measured on the 96-well plate reader Synergy 2 (BioTek, Winooski, VT, US). Fluorescence emission and excitation spectra were determined on the PC1 fluorometer (ISS, Champaign IL) and UV-vis absorption spectra were measured on a single beam spectrophotometer (Cary-50 Varian). Samples for fluorescence or absorbance measurements were prepared by dissolving 30 $\mu g\, mL^{-1}$ of C-PC in a 50 mM phosphate buffer pH 7.0. Excitation spectra were measured through a 700 nm cut-off filter and the emission spectra were recorded using 620 nm excitation and 0.5 mm width slits.

Table 1
Cyanobacterial strains that showed high content of phycocyanin, their origin, biomass yield, and phycocyanin purity in a crude extract, based on A615/280 ratio. Percent of C-PC content in biomass was calculated in a crude extract by using spectrophotometric method and it is based on measurements of three separate extractions.

Genus	Strain	Origin	Biomass yield ($g\, L^{-1}$)	% PC in biomass	615/280 ratio
Aphanotheca	80-12a	Lake Ontario, NY	1.05 ± 0.10	4.0	0.49
Anabaena	40-3	Lake Seminole, FL	0.70 ± 0.06	1.0	0.46
Anabaena	66-2	Lake Seminole, FL	0.60 ± 0.05	8.0	1.10
Limnothrix	39-1	Lake Dora, FL	1.00 ± 0.08	4.7	0.71
Limnothrix	37-2-1	Crescent Lake, FL	1.20 ± 0.10	18.0	2.08
Limnothrix	48-1	Lake Ariana, FL	0.59 ± 0.06	2.5	0.35
Limnothrix	21-2-2	STA-1W[a]	0.50 ± 0.04	5.6	0.50
Lyngbya	15-2	C111[b]	0.60 ± 0.04	5.0	0.61
Nostoc	47-3	Lake Placid, FL	0.45 ± 0.05	4.0	0.35
Synechococcus	21-11	STA-1 W	0.35 ± 0.02	5.2	0.40

[a] Storm water treatment area 1 W, FL.
[b] Everglades C111 canal, FL.