Some properties of C-phycocyanin from a native strain *Anabaena affinis* isolated from Kovada Lake, Isparta, Turkey

Aylin Akoglu*, Doruk Engin² & M. Lutfu Cakmakci³

¹Dept. of Gastronomy and Culinary Arts, Abant Izzet Baysal University, Golkoy, 14000 Bolu, Turkey
²Institute of Biotechnology, Ankara University, Beseler, 06500 Ankara, Turkey
³Dept. of Food Engineering, Ankara University, Diskapi, 06110 Ankara, Turkey

[E-mail: aylinsen61@yahoo.com]

Received 13 November 2013; revised 13 December 2013

Some properties of C-Phycocyanin (C-PC) from a native strain *Anabaena affinis* isolated from Kovada Lake, Isparta, Turkey were discussed in the present. Purification process was resulted in analytical grade product with 6.93-fold increase in phycocyanin purity. By using UV-VIS and fluorescence spectrophotometry, the highest absorbance and fluorescence were determined as 620 and 646 nm, respectively. SDS-PAGE analysis revealed the presence of two major bands with a molecular mass of 17.1 and 18.6 kDa corresponding to the α and β subunits and as confirmed by MALDI-TOF mass spectrometric analysis. Molecular mass of pure C-PC was found as 156.2 and 198 kDa by HPLC gel filtration. Aggregation states of C-PC were estimated as hexameric-(αβ), along with two binding polypeptides of 27 kDa. High antioxidant activity was determined at 480 µM TEAC/mg phycocyanin while no antimicrobial activity was detected.

[Key words: Phycocyanin, cyanobacteria, *Anabaena affinis*, purification, antioxidant activity]

Introduction

Phycobiliproteins which are brilliantly colored and water-soluble proteins are the major photosynthetic accessory pigments in cyanobacteria¹. Among the phycobiliproteins, C-Phycocyanin (C-PC) is the major component, which gives many cyanobacteria their bluish color²⁵. C-PC is the most important natural blue pigment for the food industry, as additives in chewing gum, candies, soft drinks, dairy products and jelly.²⁵ C-PC is also used as natural coloring in cosmetics and pharmaceuticals, particularly replacing current synthetic pigments which are generally toxic or unsafe.²⁶⁷ C-PC has drawn attention due to its therapeutic properties such as antioxidant, anti-inflammatory and anti-cancer activities²⁵.²⁶⁷⁸ In addition, it has also been used as fluorescent imaging tag for cells and various macromolecules in the fields of biomedical research, diagnostics, therapeutics.¹⁰⁸¹¹ In the last decades, there has been increasing interest in the potential uses of C-PC.²

The C-PC purity is expressed as the ratio of absorbances at 620 and 280 nm (A620/A280). A ratio of 0.7 is considered as food grade, 3.9 as reagent grade and greater than 4.0 as analytical grade.¹² In most studies, analytical grade C-PC was obtained using purification process including three or four purification steps and at least two chromatography techniques.¹³ Although various purification methods involving a combination of several techniques such as centrifugation, ammonium sulfate precipitation, ultrafiltration, gel filtration chromatography, ion exchange chromatography, hydroxyapatite chromatography, aqueous two phase extraction and expanded bed adsorption chromatography, have been described, there is not yet a standard technique for extraction and purification of C-PC⁴,⁵,⁹,¹⁴. Furthermore, a method which is suitable for one organism may not be appropriate for another one.¹⁵

*Spirulina platensis* is considered as host for C-PC production because of particular qualities of its C-PC.² In many studies, phycocyanin has been successfully extracted and purified from a number of organisms such as *Spirulina fusiformis*, *Calothrix* sp.⁴, *Anabaena marina*,⁹ *S. maxima*,¹² *Aphanizomenon flos-aquae*,¹⁶ *Nostoc* sp.¹⁷, *Synechococcus* sp.¹⁸, *Oscillatoria quadripunctulata*¹⁹ and *Phormidium ceylanicum* alternatively *S. platensis*. However, as well known, very few species of cyanobacteria exhibit the ability to produce high amount of C-PC. Therefore it is important to find more productive strains. Although *Anabaena affinis* is one of the most abundant cyanobacteria, no reports are available about the purification of C-PC. In the present study, *Anabaena affinis* is a native strain was used for production of C-PC and some of C-PC properties were investigated. To our best knowledge, this study is the first report concerning purification and characterization of C-PC from *Anabaena affinis*.

Materials and Methods

*Anabaena affinis* used in the present work was previously isolated from Kovada Lake, Isparta, Turkey.²¹ The strain was grown in 2 L
Erlenmeyer flasks containing 1 L of BG11 medium22 at 28°C for 25 days under 36W white fluorescent lamp illumination (130 µmol photon m⁻² s⁻¹) with a dark:light cycle of 12:12 h in shaking incubator at 120 rpm.

Twenty-five days grown cyanobacterial cells were harvested by centrifugation at 20,000xg for 10 min (Sigma 3k30, Germany) at 4°C. One volume of cell mass was resuspended in one volume of the 0.1 mol L⁻¹ Na-phosphate buffer (pH 6.8) and subjected to repeated freeze–thaw cycles of 21°C and 4°C for three times in order to release phycoerythrin. Suspension was left at 4°C for overnight and then the cell debris was removed by centrifugation at 20,000xg for 20 min. The blue supernatant of the crude extract was used in the following purification steps. All the purification steps were performed at 4°C and all the buffers used contained 1 mmol L⁻¹ sodium azide to prevent the contamination.

The crude extract of C-PC was fractionated by precipitation with solid ammonium sulfate first at 25% and then at 75% saturation. Finely powdered ammonium sulfate was gradually added into the crude extract until achieving 25% saturation was reached, with continuous stirring for 1 h. The resulting solution was kept for 4 h and centrifuged at 20,000 x g for 20 min. The supernatant was pooled and subjected to 75% ammonium sulfate saturation in a manner similar to that of 25% saturation. After overnight incubation, the solution was centrifuged at 20,000 x g for 20 min. The precipitated C-PC was dissolved in a small volume of 10 mmol L⁻¹ Na-phosphate buffer (pH 6.8) and dialyzed for 48 h against 100 times volume of the same buffer, with four times buffer exchange.

Six milliliter crude extract was filled in the ultrafiltration tube with 100 kDa molecular weight cutoff rated polyethersulphone ultrafiltration membrane (Vivaspin6, Sartorius, Germany) and centrifugated at 4,000 x g for 1 h.

CaptoQ (General Electric Healthcare, USA) strong anion exchange column (50 mm bed height x 16 mm internal diameter, 10 mL column bed volume) was pre-equilibrated with 25 mmol L⁻¹ Na-phosphate buffer (pH 7.0). Afterwards, 10 mL of suspension was filled into the column (BioLogic DuoFlow 10, USA). Following washing with 10 column volumes of the same buffer, linear gradient elution was performed with 10 column volumes of 0 to 0.6 mol L⁻¹ NaCl in 25 mmol L⁻¹ Na-phosphate buffer (pH 7.0) at a flow rate of 2 mL min⁻¹. Blue-coloured fractions were collected and each fraction was analyzed by using UV-Vis spectroscopy and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Analytical Procedures

Analytical calculations were made according to Antelo et al.23. The C-PC concentration in the sample was calculated using Equation 1. A₆₂₀ and A₆₅₂ were determined to be the optical densities of the sample at 620 nm and at 652 nm, respectively. The absorption spectrum was measured on a Shimadzu UV-1700 spectrophotometer (USA). The purity ratio of the C-PC was determined by using A₆₅₀/A₆₈₀ ratio. Purity ratio was taken as a criterion for phycoerythrin purity achieved at each step of purification.

\[ \text{Purity ratio} = \frac{A_{650}}{A_{680}} \]

The recovery efficiency of the C-PC was calculated using Equation 2. C-PC_{AP} denotes the amount of phycoerythrin after the purification when C-PC_{BP} presents that of phycoerythrin before the purification.

\[ \text{Recovery} \% = \frac{\text{C-PC}_{AP}}{\text{C-PC}_{BP}} \times 100 \]

Purification factor was found by Equation 3. The abbreviation of AP displays the purity ratio of C-PC after the purification and BP is attributed to the purity ratio of the crude extract.

\[ \text{Purification factor} = \frac{\text{Purity ratio}_{AP}}{\text{Purity ratio}_{BP}} \]

Some properties of C-PC

Denaturing gel electrophoresis of C-PC was carried out by using 8x7 cm slabs of 15% acrylamide in separating and 4% in stacking gels. Partially or fully purified C-PC samples were mixed with 2x gel loading buffer which contained 20 g L⁻¹ SDS, 100 mL L⁻¹ glycerol, 45 mL L⁻¹ β-mercaptoethanol, 0.25 g L⁻¹ bromophenol blue and 60 mmol L⁻¹ Tris-Cl (pH 6.8) at a ratio of 1 : 1. After incubation for 10 min in a boiling water bath, 25 µL of the sample mixture and 10 µL of prestained molecular mass standard (Fermentas, SM0661, Lithuania) were loaded onto the gel. Gels were run at room temperature using a Laemmli buffer system24 at fixed current of 25 mA for the first hour and then the current was shifted to 35 mA until the end of run. Gels were stained by using Coomassie brilliant blue G-250 (Merck, Darmstadt, Germany). The molecular weights of subunits were determined by calibrating the gel with molecular weight markers between 10-200 kDa (SM0661, Fermentas, UK).

Spots were carefully cut out from colloidal Coomassie stained gels and in-gel digestion with trypsin was performed according to Shevchenko et al.25 with some minor modifications. All the
steps of in-gel digestion were performed in the laminar flow cabinet to avoid contamination. Peptides were resolubilized, spotted onto a target plate and dried. Wells were analyzed and the spectra were recorded by MALDI-TOF mass spectrometer (Micromass, Waters, UK) operated in positive-ion reflectron mode for the mass range 500-3,000 Da. The raw spectra were analyzed using MassLynx 4.0 software. Due to the peak clusters originating from matrix, parts of spectra below 700 Da were overlooked. Protein identification was performed by searching in a protein sequence database (NCBI-National Center for Biotechnology Information) using MASCOT program (http://www.matrixscience.com) and SwissProt /UniprotKB (http://www.uniprot.org) database. C-PC structures belong to different microorganisms were determined in previous studies which were located in databases. These C-PC structures were applied unreal tryptic cutting and results were compared with experimental m/z values.

**Determination of molecular weight by high performance liquid chromatography (HPLC)**

HPLC was performed to determine the molecular weight and estimate the aggregation states of intact C-PC. TSKGel filtration column (G2000SWXL, TOSOH Corporation, Japan) was connected to high performance liquid chromatography work station (Hp Agilent 1200, USA). Sodium phosphate buffer (pH 7.0) at a concentration of 25 mmol L\(^{-1}\) was used as mobile phase and flow rate was maintained at 1 mL min\(^{-1}\). UV-Vis Spectrometric detector channels were set to monitor absorbances at wavelengths of 280 and 640 nm, in order to detect total protein and C-PC respectively. After preconditioning the column, molecular weight standard which contained myoglobin (215 kDa), phosphorylase B (120 kDa), bovine serum albumin (84 kDa), ovalbumin (60 kDa), carbonic anhydrase (39.2 kDa), trypsin inhibitor (28 kDa), lysozyme (18.3 kDa) (Pierce Cat#26681, USA) was run in triplicate to obtain standard curve for column retention times for the given range of proteins. Fifty microliters of purified C-PC was injected to the column. Molecular weight of C-PC was calculated by using the regression curve (R\(^2\) value was 0.9983) obtained from the molecular weight standard.

Fluorescence spectrum of purified C-PC was determined by using a multimode plate reader (Spectramax M4, Molecular Devices, USA). Firstly, C-PC sample was excited with a fixed wavelength light of 480 nm, emission readings were performed at 590 to 760 nm with 2 nm increments. Next, C-PC sample was excited with 500 to 680 nm monochromatic light source with 2 nm increments. Emissions were read at 720 nm. Fluorescence spectrum was constructed by using normalized excitation and emission data.

For the antibacterial activity test, *Escherichia coli* ATCC 25295, *Salmonella enterica* serotype Typhimurium SL 1344, *Staphylococcus aureus* ATCC 6538, *Listeria monocytogenes* ATCC 7644, *Enterococcus faecium* ATCC 6057, *Bacillus subtilis* ATCC 21332 cultures obtained from the culture collections of Biology Department of Ankara University were used. Overnight cultures were grown in convenient media and temperature. Antibacterial activity was carried out using spot on law method. 10 μL C-PC of was inoculated on agar surface including pathogen microorganism and plates were incubated at 37°C for 24 h. After the incubation, the diameter of inhibitory zone surrounding spot measured in mm. The standard TEAC (Trolox Equivalent Antioxidant Capacity) assay described by Van den Berg et al. and by Re et al. was used with minor modifications for determination of the TEAC value. This assay assesses the total radical scavenging capacity based on the ability of a compound to scavenge the stable ABTS \([2, 2′-\text{Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)}\] radical (ABTS\(^-\)) in 6 min. The bluegreen ABTS\(^-\) is generated through the reaction between 7 mm ABTS and 2.45 mm potassium persulfate in water. This solution was stored in the dark for 12–16 h before use. The concentrated ABTS\(^-\) solution was diluted with phosphate buffered saline (PBS) to give a final absorbance of 0.7±0.02 at 734 nm. Stock solutions of trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) served as a standard were prepared in ethanol. Ten microliters of an antioxidant containing solution were added to 990 ml ABTS\(^-\) solution and the absorbance at 734 nm was measured in time. This was compared to a blank where 10 ml of the solvent was added to 990 ml of the ABTS\(^-\) solution. The reduction in absorbance 6 min after addition of the antioxidant was determined. The TEAC of the antioxidant was calculated by relating this decrease in absorbance to that of a trolox solution on a molar basis.

**Results and Discussion**

Growth pattern in terms of C-PC concentration of *Anabaena affinis* was drawn in order to clarify the optimal incubation period. It is visible from the Fig. 1 that the cultivation period of 25 days was determined as optimum for C-PC production.
with 36.03 mg mL\(^{-1}\) (110.52 mg g\(^{-1}\)) C-PC concentration. In some researches, C-PC concentrations were determined as 77.0 mg g\(^{-1}\) from \textit{S. maxima}\(^{30}\) 82.48 mg g\(^{-1}\) from \textit{S. platensis} \(^{31}\), 46.8 mg g\(^{-1}\) from \textit{S. platensis} \(^{32}\), 72.5 mg g\(^{-1}\) from \textit{Anabaena} sp. ATCC 33047 \(^{33}\). It is associated with the fact that the \textit{Anabaena affinis} is more productive strain as compared to several cyanobacteria strains. Therefore, it is not wrong to verify that the \textit{Anabaena affinis} strain is available and alternative for the production of the C-PC instead of \textit{S. platensis} (the ancestor of the C-PC producer). In the present work, the C-PC extracted from the \textit{Anabaena affinis} strain was purified and characterized for future studies of the product on food industry, cosmetics and pharmaceuticals.

Results of all purification steps were shown in Table 1. The various ammonium sulfate saturation concentrations (30\% + 50\%, 25\% + 75\%, 70\% and 80\%) were tested and the results obtained showed that maximum purity and recovery values of the C-PC product were observed within two-step precipitation viz. 25\% and 75\% ammonium sulfate precipitation (data not shown). With the aid of the ASP method, we were able to achieve C-PC product with 1.96 purity ratio, 79.93\% recovery and 3.38 purity factor. Based on the findings, it is natural to confirm that the unique two steps in the ASP method outperformed a number of reports in terms of the purity ratio and recovery values of the C-PC sample \(^{15,34\&35}\). Similar to our results \(^{1}\) obtained are also in good agreement with the previous reports given. This is attributed to both the properties of producer strain and determination of optimum UF conditions.

Elution obtained from UF was loaded to ion exchange chromatography. Blue colored fractions collected at 50-70 mins were used to check the quantity and purity of the C-PC sample. With the aid of the IEC method 1.65-fold enhancement in the C-PC purity were observed. The results obtained are also in good agreement with the previous reports given. Strong cation exchanger column was used for IEC, differently from previous studies. Thus, it would be more precise to say that the performance of the strong cation exchanger column used in this work is better as compared to the weak cation exchangers used in earlier studies \(^{17,18,34\&35}\). Even, nobody encounters any reports based on the strong cation exchanger column in the previous studies. Based on the findings, purification process including the ammonium sulfate precipitation, ultrafiltration and ion exchange chromatography in which the sample was produced with the purity factor of 6.93 and purity ratio of 4.02 exhibits the most efficient process. Besides, the analytical grade product was obtained at the end of this process.

![Fig. 1 — Growth pattern in terms of C-PC concentration in Anabaena affinis of 0–35 days of incubation](image)

Some properties of purified C-PC

SDS-PAGE analysis revealed two major bands at 17.1 and 18.6 kDa, corresponding to \(\alpha\) and \(\beta\) subunits of C-PC, respectively (Fig. 2). According to this result, the molecular weight of monomer was found to be 35.7 kDa. One can see the similar results in the literature where the molecular weight of monomer was determined as 36 kDa \(^{36}\). However; it is possible to encounter the greater molecular weight belonging to the

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Purity ratio</th>
<th>Recovery (%)</th>
<th>Purity factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>0.58</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate precipitation</td>
<td>1.96</td>
<td>79.93</td>
<td>3.38</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>2.43</td>
<td>62.18</td>
<td>4.19</td>
</tr>
<tr>
<td>Ion exchange chromatography</td>
<td>4.02</td>
<td>18.56</td>
<td>6.93</td>
</tr>
</tbody>
</table>
monomer in the literature. Peptide mass fingerprinting was exerted to further identify the protein bands obtained in SDS-PAGE. Mass spectra inferred from the tryptic digest of α and β subunit bands were analyzed and peptide sequences were deduced in detail. *Anabaena affinis* peptide sequences obtained from previous study were extracted from SwissProt and UniprotKB protein databases. Blast searches performed in these databases returned similar protein sequences with mean coupling percent (data not shown). MALDI-TOF analysis confirmed that the protein bands obtained from SDS-PAGE belonged to the C-PC subunits. Additionally, the capillary electrophoresis or HPLC-coupled mass spectrometric analysis is suggested to perform more extensive and conclusive analysis of the C-PC purified.

Fig. 2—SDS-PAGE of purified C-PC. L1 Molecular mass marker; L2 Crude extract; L3 Purified C-PC

Two different peaks corresponding to 156.2 and 198 kDa were detected for the pure C-PC in HPLC gel filtration. The aggregation state of 198 kDa peak was estimated as the hexameric form-((αβ)_6) whereas the trimeric form-((αβ)_3) with two 27 kDa linker protein was assigned to 156.2 kDa peak. From the peak areas in the chromatogram as shown in Figure 3, the relative abundances of hexameric and trimeric forms were computed to be 21% and 79% of total C-PC, respectively. Furthermore, it is necessary to underline that the aggregation state of phycobiliproteins strictly depends on pH, ionic strength and protein concentration. Purification process in the present study was carried out in pH 7.0. Most common aggregation state is related to the trimeric form at neutral pH with the moderate ionic strength and protein concentration. Molecular structure can also change with regard to the type of microorganism. Distinct forms demonstrated in this comprehensive study may have an impact on physical and biochemical properties of C-PC.

Fig. 3—Chromatogram of HPLC

Spectral scanning of the purified C-PC displayed a sharp absorbance peak at 620 nm with concomitant decrease in absorbance at 280 nm, confirming the successful removal of proteins except for those of the C-PC. Notably, no absorbance peak was detected at either 650 nm or 540 nm. This is attributed to the absence of phycoerythrin or allophycocyanin in the purified product (Fig. 4). A fluorescence excitation-emission spectrum of the purified C-PC was analyzed seriously. The fluorescence spectrum of purified C-PC exhibited the maximum emission at 646 nm (Fig. 5). This result observed is in good agreement with the fluorescence property of the C-PC specimen reported in the literatures.

Fig. 4—UV–Vis absorption overlay spectra of C-PC at each step of purification. ---, Crude extract; ——, Ultrafiltration elute; ——, Ion exchange chromatography elute

Pure C-PC was tested for its antibacterial activity against six different bacterial cultures: *Escherichia coli* ATCC 25295, *Salmonella enterica* serotype Typhimurium SL 1344, *Staphylococcus aureus* ATCC 6538, *Listeria monocytogenes* ATCC 7644, *Enterococcus faecium* ATCC 6057, *Bacillus subtilis* ATCC 21332. Sabarinathan and Ganesan obtained C-PC from filamentous fresh water cyanobacterium *Westiellopsis* sps. and detected antibacterial activity of C-PC against three different bacterial cultures: *Bacillus subtilis*, *Pseudomonas* sp. and *Xanthomonas* sp.. On the contrary, no inhibition
zone and hence no antibacterial activity was detected against six different bacterial cultures in the present study.

The antioxidant activity of phycocyanin was assayed by ABTS\textsuperscript{+} method and it was expressed as Trolox Equivalent Antioxidant Capacity. Due to the linear tetrapyrrolo prosthetic group commonly called as “bilins” of phycocyanin, molecule has ability to scavenge reactive oxygen species and can behave as an antioxidant\textsuperscript{10}. In the present study, the results showed that C-PC had 480 µM TEAC mg\textsuperscript{-1} phycocyanin and this value was quite high. Leelapornpisid \textit{et al}.\textsuperscript{9} informed that antioxidant activity of C-PC obtained from \textit{S. platensis} was 48.5 µM TEAC mg\textsuperscript{-1} phycocyanin. It was tough that, this difference can be related that aggregation form and purity value of C-PC obtained from different strain. The result suggesting the potential use of phycocyanin obtained from \textit{Anabaena affinis} as an antioxidant protein in various preparations.

**Conclusions**

The findings deduced from this comprehensive work indicated that the \textit{Anabaena affinis} strain is available and alternative for the production of the C-PC. Last values obtained from UF method are larger than the values in the literature as a consequence of the effect of the producer strain and especially determination of optimum conditions. As a matter of structure, our result indicated that structure of C-PC obtained from \textit{Anabaena affinis} was different from other previous studies reported. Moreover, our purified C-PC is having potential role as an antioxidant compound. However further detailed studies on structure and stability of C-PC are needed to be evaluated.

**Acknowledgements**

Authors would like to thank Asst. Prof. Dr. Fatma Gürbüz for providing the strain \textit{Anabaena affinis} and Dr. Beycan Ayhan for helping with MALDI-TOF analysis and also thank Prof. Mustafa Akçelik and staff in the laboratory at Ankara University, Biotechnology Institute for their help in protein purification and characterization.

**References**