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Different characteristics of C-phycocyanin (C-PC) in two strains of the extremophilic *Galdieria phlegrea*

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ABSTRACT

To date, the main source for phycocyanin production is the thermophilic cyanobacterium *Spirulina platensis*, although latest researches are exploring the possibility to exploit the thermoacidophilic Cyanidiophyceae (Rhodophyta) to this purpose. *Galdieria phlegrea* is a polyextremophilic red alga belonging to Cyanidiophyceae, colonizing, along with *G. sulphuraria*, acidic and thermal environments and distinguishable from this latter on the base of molecular and ecophysiological traits. In the present paper, a characterization of C-phycocyanin from two strains of *G. phlegrea* with different geographic provenance (Phlegrean Fields Naples, Italy; Diyadin, Turkey) was provided under autotrophic and heterotrophic conditions. The results showed different optimal pHs and thermostability between the strains and between autotrophic and heterotrophic cells. What is intriguing, a pre-heating at 70 °C of heterotrophic cells from the Italian strain resulted in a highly heat-resistant of C-phycocyanin. We hypothesized that the different features of this pigment, currently widely used for various applicative purposes, are related to the habitat from which the microalga comes from.

1. Introduction

C-phycocyanin (C-PC) belongs to a group of light harvesting proteins named phycobiliproteins and it is a major photosynthetic pigment of Cyanobacteria, Rhodophyta and Cryptophyta [1]. C-PC is a water-soluble and highly stable chromoprotein (or biliprotein, 70,000–110,000 Da), composed by apoproteins covalently bound to phycocianobilin chromophore; absorbance maxima is at 615–630 nm (where chlorophyll *a* has a low absorptivity) and fluorescence emission maxima at approximately 640 nm. The phycobiliprotein consists of two relatively homologous subunits, α and β, in a 1:1 ratio, the former with one phycocyanobilin bound at cys84, the latter with two phycocyanobilins bound at cys84 and 155 [2]. The C-PC has a distinctive deep blue colour and, thanks to its fluorescent properties, C-PC is employed as fluorescent tags in clinical and immunological laboratories and in fluorescent microscopy [3]. Its antioxidant and anti-inflammatory properties make C-PC a safer ingredient for food, nutraceutical and pharmaceutical purposes [4].

The thermophilic cyanobacterium *Spirulina platensis* is up-to-date the main source of C-PC for industrial destination, although its production suffers of limitations due to bacterial and fungal contamination, which affect the productivity as well the quality of the pigment. An interesting alternative is offered by the thermoacidophilic Cyanidiophyceae (Rhodophyta), one of most ancient groups of algae [5,6] widely distributed across most of the geothermal areas throughout the Earth [7–10]; their emerald colour derives from a combination of chlorophyll *a* and phycobiliproteins (C-PC, allophycocyanin and phycoerythrin), accessory pigments joint in the phycobilisomes and lodged on the thylakoids. Cyanidiophyceae are well adapted to the environmental extremes offered by the volcanic areas, where temperatures rise above 50 °C, and high sulphuric acid concentrations greatly reduce the pH to values prohibitive to the majority of eukaryotic life forms (pH 0.5–3.0). Among Cyanidiophyceae, *Galdieria sulphuraria* possesses some peculiar aspects, which makes it a good candidate to substitute *Spirulina* in the production of C-PC: it is able to grow mixo- and heterotrophically fast on a wide number of carbon sources [11] and several strains are capable to preserve their photosynthetic apparatus when grown heterotrophically [11–13]. Moreover, a C-PC with a high thermostability was recently isolated and characterized from *G. sulphuraria* [2]. A highly thermostable C-PC was also recently isolated by

Abbreviations: C-PC, C-phycocyanin; ACUF, Algal Collection at University Federico II, Naples; ODλ, optical density at a certain wavelength λ

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Cyanidioschyzon merolae [14], another thermoacidotolerant member of Cyanidiophyceae. However, despite the high thermostability of C-PC and the absence of a cell wall that would facilitate the extraction of C-PC from *C. merolae*, the heterotrophic ability and the acidophily of many *Galdieria* strains make them possible candidates to replace *Spirulina* in the production of C-PC. *Galdieria phlegrea* is a new species recorded for the first time in cryptoendolithic environments located in the Phlegrean Fields (Naples, Italy), adapted to relatively dry conditions and to reduced light intensities [7,8]. *G. phlegrea* possesses interesting ecophysiological traits, exhibiting growth rates at 25 and 38 °C lower than the *G. sulphuraria* as well as lower photosynthetic rates confirming its low-light adaptation. Moreover, although *G. sulphuraria* and *G. phlegrea* share a significant common gene pool, comparative genomic analysis among the two species, have revealed that *G. phlegrea* have adopted ameliorative strategies for adaptation to specific environmental niches, such as the re-acquisition of a complete data-set of genes required for urea hydrolysis, necessary as an alternative nitrogen source in N-limited environments [15]. Recent explorations in Turkish thermal baths revealed the presence of *G. phlegrea* preeminently on subneutral and neutral soils, in thermal and mineralized springs, with temperatures ranging from 30 to 64 °C, and pH 6.0–7.9 [16]. The peculiar ecological and genomic traits of this species prompted us to characterize C-PC from *G. phlegrea*, under autotrophic or heterotrophic conditions. The thermostability and pH optimum of partially purified C-PC, along with the effects of heat shock on C-PC were evaluated both in the Italian and in the Turkish strain of *G. phlegrea*, in an attempt to provide new interesting insights for production and applications of C-PC from a new Cyanidiophycean species.

2. Materials and methods

2.1. Algal strains and growth conditions

G. phlegrea strains ACUF 734 (Agri Diyadin, Turky) and ACUF 009, and *G. sulphuraria* ACUF 011, were both deposited at the ACUF collection of the Department of Biology of the University of Naples "Federico II" (http://www.biologiavegetale.unina.it/acuf.html). For the experimental design, *G. phlegrea* and *G.sulphuraria* cells were grown in batches containing 1 L of Allen medium [17] under 90 μmol m^{-2} s^{-1} photons at 37 °C in a thermostatic chamber (ANGELANTONI CH 770). Heterotrophy was guaranteed by adding glycerol 2% (*w/v*) as carbon source under the dark.

2.2. Phylogenetic analyses

The molecular relation between Turkish and Italian strains of *G. phlegrea* was assessed; rbcL sequences from Turkish and Italian strains of *G. phlegrea* were aligned with published sequence data (e.g. [7–10,18,19] obtained from GenBank (www.ncbi.nlm.nih.gov), using BioEdit Sequence Alignment Editor (http://www.mbio.ncsu.edu/BioEdit/bioedit.html)). No gaps or indels have been incorporated in the alignments. Newly determined sequences were all available on NCBI GenBank. Maximum likelihood (ML) phylogenetic analysis of rbcL was performed using the GTR + Γ + I model implemented in RAxML software [20]. Statistical support for each branch was obtained from 1000 bootstrap replications using the same substitution model and RAxML program settings.

2.3. Induction of heat stress

For the heat stress experiments, aliquots of cells of *G. phlegrea* ACUF 009 (Italy) and ACUF 734 (Turkey) were harvested at logarithmic phase grown cells (approximately OD_{550} = 1.5) and transferred to 70 °C as the treatment group; other aliquots were still cultured as in precultivation conditions as control. All tests were performed in triplicates. Treated cells were kept at 70 °C for 10 min and then harvested immediately by centrifugation for 5 min at 4000*g* and used for the following determinations.

2.4. Preparation of crude extract

Control and heat-stressed cells (200 mL of culture, about 0.2 g dry weight) of both strains under auto- and heterotrophy were harvested by low speed centrifugation (4000 *g* for 5 min at 4 °C) and washed twice in H_2O distilled to remove culture medium components. The packed cells were re-suspended in 10 mL of cold extraction buffer (50 mmol L^{-1} Na-phosphate buffer, pH 7.2) and disrupted through a French pressure cell (1100 *psi*). The homogenate was centrifuged at 15,000 *g* at 4 °C for 30 min, and the clear supernatant was used as crude extract.

2.5. Purification and determination of C-PC content

Cell debris and proteins in cell crude extracts of both Italian and Turkish *G. phlegrea* were precipitated by ammonium sulphate (25–50%) at 4 °C under continuous stirring overnight. The precipitate was pelleted by centrifugation for 30 min at 15,000 *g* at 4 °C. The pellet was re-dissolved in 50 mmol L^{-1} Na-acetate buffer (pH 4.2). The C-PC content was measured at 620 and 650 nm and the concentration was determined as described by Kursar and Alberte equation [21].

In extracts the concentration of protein was determined by the Bio-Rad protein assay based on the Bradford method (1976) [22], using bovine serum albumin as the standard.

2.6. Determination of optimum temperature and pH values

To investigate the effect of various temperatures on the stability of C-PC, 1 mL of purified extract by both strains of *G. phlegrea* grown in auto- and heterotrophic conditions, was divided into several aliquots of 250 μL and diluted 4× by adding 750 μL of 50 mM Na-acetate pH 4.2 buffer. C-PC solution was incubated in micro tube at 350 rpm in an Eppendorf Thermo mixer (Eppendorf, Hamburg, Germany) for 30 min (intervals of 10 °C from 20 to 70 °C). After the heat treatments, the samples were centrifuged to remove debris and analyzed by spectrophotometer (as described above) to evaluate the C-PC residual contents. The content of C-PC (%) is the remaining concentration of C-PC as a percentage of the initial level in samples kept at room temperature (20 °C).

To determine the pH stability of C-PC, 250 μL purified extracts were incubated in 750 μL buffer solutions, with pH increasing from 1.5 to 9.0 (intervals of 0.5); each buffer solution contained sodium acetate ($C_2H_3NaO_2$) pH 1.5–5.0, Bis-Tris propane ($C_{11}H_{26}N_2O_6$) pH 5.5–7.0, Trizma® base ($C_4H_{11}NO_3$) pH 7.5–8.0, CHES ($C_8H_{17}NO_3S$) pH 8.5–9.0. The diluted extract with the different buffers was placed in cuvettes. After 5 min, at room temperature (20 °C), the concentration of pigment was analyzed with spectrophotometer, as previously described.

2.7. Statistical analysis

Experimental data analyses were carried out. Data of the mean ± standard deviation of 3–5 independent experiments were presented. The statistical analysis was performed by one-way analysis of variance (ANOVA) with a Tukey post-hoc test to determine differences between autotrophic and heterotrophic conditions and different strains.

3. Results and discussion

3.1. Phylogenetic analyses

New explorations in thermal baths located around Turkey provided the evidence of the presence of Cyanidiophycean populations on neutral/alkalyne soils [23]. All genera and species within Cyanidiophytina were collected in Turkey, including *G. phlegrea*; the inferred RAxML tree

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