Free Radical Research, August 2006; 40(8): 821–825

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healthcare

Photodynamic action of C-phycocyanins obtained from marine and fresh water cyanobacterial cultures: A comparative study using EPR spin trapping technique

BIBBIN TOM PAUL[1], ANAMIKA PATEL[2], GOVINDAN SADASIVAM SELVAM[1], SANDHYA MISHRA[2], PUSHPITA KUMAR GHOSH[2], & RAMACHANDRAN MURUGESAN[3]

[1]*Centre for Potential in Genomic Science, School of Biological Sciences, Madurai Kamaraj University, Madurai, Tamil Nadu, India,* [2]*Central Salt & Marine Chemicals Research Institute (CSMCRI), G.B. Badheka Marg, Bhavnagar, Gujarat, India, and* [3]*School of Chemistry, Madurai Kamaraj University, Madurai, Tamil Nadu, India*

Accepted by Dr B. Kalyanaraman

(Received 19 October 2005)

Abstract
C-phycocyanins, major biliproteins of blue green algae (cyanobacteria), widely used as colourants in food and cosmetics are known for their antioxidant as well as therapeutic potential. Recent claims indicating phycobiliproteins exert stronger photodynamic action on tumor cells than clinically approved hematoporphyrin derivatives motivate us to investigate the photodynamic action of two newly isolated C-phycocyanins from *Phormidium* [PHR] and *Lyngbya* [LY] *spp*, respectively in comparison with known C-phycocyanin from *Spirulina sp.* [SPI]. Photolysis of air saturated solutions of PHR, LY and SPI in the presence of 2,2,6,6-Tetramethyl piperidinol (TEMPL) generated three line EPR spectrum characteristic of 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxyl (TEMPOL). The increase in intensity of the EPR spectrum with time of irradiation and decrease in intensity, in the presence of $^1O_2$ quencher DABCO confirm the formation of $^1O_2$. Photoirradiation in the presence of spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) generated EPR signal characteristic of O_2^- adduct. Efficiency of $^1O_2$ generation is of the order LY > PHR > SPI. The yield of reactive oxygen species (ROS) generation is found to be $^1O_2 > O_2^-$ indicating type II mechanism to be the prominent pathway for photosensitation by phycocyanins.

Keywords: *Blue green algae, C-phycocyanin, 2,2,6,6-tetramethyl piperidinol, 5,5-dimethyl-1-pyrroline-N-oxide*

Introduction

Phycobiliproteins are water soluble fluorescent proteins derived from cyanobacteria and eukaryotic algae. These proteins are composed of a number of subunits, each having a protein backbone to which linear tetrapyrrole chromophores are covalently bound [1]. Phycocyanins, the blue coloured phycobiliproteins have absorption maxima between 610 and 665 nm, and are of greater importance, because of their various biological and pharmacological properties. It is claimed that phycobiliproteins exert stronger photodynamic action on tumor cells than clinically approved hematoporphyrin derivatives [2]. Therefore, phycocyanins may be of use as a novel second generation photosensitizer for photodynamic therapy (PDT), a minimally invasive therapeutic approach for the management of a variety of tumors and certain benign diseases. PDT is based on the combined use of a light-absorbing compound (photosensitizer), which accumulates preferentially in cancerous tissue, and visible light irradiation of a wavelength matching the absorption spectrum of the photosensitizer [3]. The excited states of the photosensitizer participate either in a one-electron oxidation-reduction reaction (type I

Correspondence: R. Murugesan, School of Chemistry, Madurai Kamaraj University, Madurai 625021, India. Tel: 91 452 2458246. Fax: 91 452 2459105. E-mail: rammku@eth.net

ISSN 1071-5762 print/ISSN 1029-2470 online © 2006 Informa UK Ltd
DOI: 10.1080/10715760600696938

photochemistry), generating oxygen free radical, or energy transfer to ground state oxygen (type II photochemistry), generating singlet oxygen. Reactive oxygen species (ROS) generated by the photosensitizer make irreversible cell damage to the tumor cell eventually leading to the cell death.

Phycocyanins exhibit several advantages over the presently used hematoporphyrin derivatives. Some of which include (1) ready preparation and easy purification, (2) good absorption in the phototherapeutic window (600–900 nm), (3) high molar exitinction coefficients and (4) minimal side effect and significant reduction in normal tissue photosensitivity. Presence of phycobiliproteins is one of the means by which cyanobacteria can exist in a diversity of ecological niches such as polar regions, thermal hot springs and hypersaline ecosystem and consequently thrive under vastly different environmental stress [4]. Phycocyanins isolated from cyanobacterial species from various environmental niches like, marine (*Phormidium* and *Lyngbya spp.*), fresh water (*Spirulina sp.*) etc have shown differences in certain properties such as structural stability and antioxidant activity [5]. Early report on photogeneration of $^1O_2$ by phycobiliproteins comes from indirect method either by checking generation of DMPO-OH as follows

$$^1O_2 + \text{DMPO} \rightarrow [\text{DMPO} -{}^1 O_2] \overset{H_3O^+}{\rightarrow} \text{DMPO}-\text{OH} + \text{OH}^{\cdot}$$

$$\text{OH}^{\cdot} + \text{DMPO} \rightarrow \text{DMPO}-\text{OH}$$

or by spectroscopically monitoring photobleaching of phycobiliproteins by the generated $^1O_2$ [6,7]. Here, we used a direct and more decisive EPR- method [TEMPL-assay] to study photodynamic generation of $^1O_2$ by three phycocyanins. Our work further aims to investigate whether, changes in properties of phycocyanins, isolated from different sources, are reflected in their photodynamic action, and to make a comparative study of the ROS generation efficiency of three phycocyanins using EPR-spin trapping technique.

Materials and methods

Chemicals

Superoxide dismutase (SOD) was purchased from Sigma chemical Co. Dimethyl sulphoxide (HPLC grade) was procured from Qualigens Fine Chemicals, India. The spin trap 5,5-Dimethyl −1-pyrroline-N-oxide (DMPO) was obtained from Aldrich and was purified by activated charcoal [8]. 2,2,6,6-Tetramethyl piperidinol (TEMPL) was obtained from Merck, India. 1,4-diazabicyclo[2,2,2]octane (DABCO) was purchased from Aldrich Chemicals Co.

Isolation and purification of C-PC

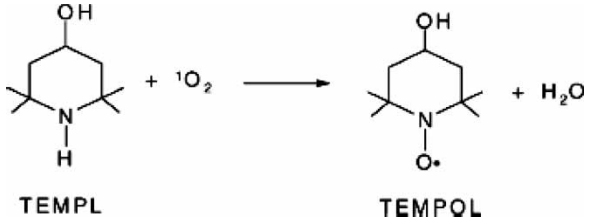
The marine cyanobacterium *Phormidium* and *Lyngbya spp.* were isolated from the rocky surface near the sea coast of Gujarat (Lat. 21°38′ N and Long. 69°37′), the west coast of India. These organisms were grown in batch cultures in the standard artificial seawater medium ASN-III [9] at pH 7.5 and temperature 20 ± 2°C with optimum light intensity of 60 μ E m^{-2} s^{-1}, provided by cool-white fluorescent tubes with a dark/light cycle of 12:12 h. Fresh water cyanobacterium *Spirulina sp.* was grown in batch cultures in Zarrouk's medium [10] at pH 10 and temperature 20 ± 2°C with optimum light intensity of 60 μ E m^{-2} s^{-1}, provided by cool-white fluorescent tubes with a dark/light cycle of 12:12 h. C-PC from *Spirulina, Phormidium* and *Lyngbya spp.* were extracted and purified as described earlier [11].

Light source

Light source used for irradiation was a 150 W xenon lamp. A filter combination of 10 cm potassium iodide solution (1 g in 100 ml) and 1 cm pyridine was used to cut off light below 300 nm and achieve a spectral window of 300–700 nm. The irradiation was generally carried out in an open cuvette, in equilibrium with the atmosphere. Reaction mixture was kept at a distance of 12 cm from the light source and constantly stirred.

Detection of reactive oxygen species by EPR -spin trapping

Detection of singlet oxygen. The photogeneration of singlet oxygen by phycocyanins was investigated by EPR- spin trapping [12,13]. The experiment was carried out in a mixture (1 ml) containing 0.02 M TEMPL and phycocyanin (2 mg/ml) in DMSO. The mixture was irradiated and the increase in EPR signal was followed as a function of time. TEMPOL, a stable nitroxide free radical, formed as a result of oxidation of TEMPL by $^1O_2$ (as given below) shows a three-line EPR-spectrum.



The formation of TEMPOL was not observed in dark. EPR- spectra were measured using a JEOL JES-TE100 ESR spectrometer. Samples were injected into gas-permeable Teflon capillary tube (0.8 mm inside diameter, 0.5 mm wall thickness), which was folded

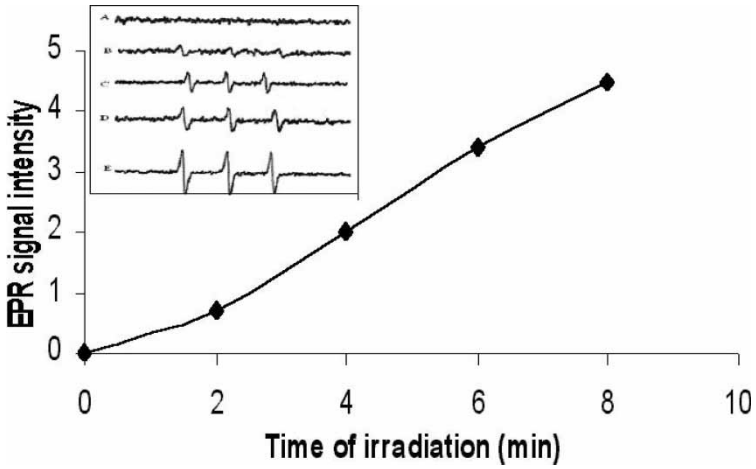


Figure 1. Plot showing EPR signal intensity for singlet oxygen generation of phycocyanin isolated from *Lyngbya sp.* vs time of irradiation. The inset shows the EPR spectra of TEMPOL generated during the photoirradiation of DMSO solution of phycocyanin PHR (2 mg/ml) in the presence of TEMPL (20 mM) at 300 K. (A) in the dark, (B) 2-min irradiation, (C) 4-min irradiation and (D) 6-min irradiation (E) 8-min irradiation. Spectrometer setting: microwave power, 2 mW; modulation frequency, 100 kHz; modulation amplitude, 0.5; time constant, 0.1 s; scan rate, 4 min; scan width, 200 G; receiver gain, 500.

and inserted into a narrow quartz tube and placed in the EPR cavity for measurements [14,15].

Detection of superoxide anion. Photogeneration of superoxide anion by phycocyanin was also investigated by EPR- spin trapping technique [16,17]. Solution of phycocyanin (2 mg/ml) and DMPO (100 mM) in DMSO was irradiated in a quartz cuvette, which was continuously stirred using a magnetic stirrer during irradiation. Irradiated solution was drawn into a gas-permeable Teflon capillary tube and placed in the EPR- cavity. EPR- spectra were measured at room temperature.

Results and discussion

Generation of singlet oxygen

Singlet oxygen is believed to be the major mediator of photochemical cell damage for many types of photosensitizers [18]. Hence, we have investigated the photogeneration of $^1O_2$ by EPR technique because EPR is considered the least ambiguous method for detection of free radicals [19]. EPR- spectra, characteristic of TEMPOL nitroxide radical, were observed when aerated solution of phycocyanins (PHR, SPI and LY) and TEMPL in DMSO were irradiated at room temperature. The hfcc (A_N = 15.7 G) was found to be identical with those of authentic TEMPOL sample. EPR signal intensity of TEMPOL was found to increase with increase of irradiation time as shown in Figure 1. Other compounds also showed similar behaviour. Control experiments indicated that sensitizer, oxygen and light were all essential for the production of TEMPOL, indicating that the formation of nitroxide radical is a photodynamic process. The rate of formation of TEMPOL by phycocyanins SPI, PHR and LY are related to their $^1O_2$ generating efficiency. Comparative plot of EPR signal intensity against time by various phycocyanins as shown in Figure 2 shows the order of $^1O_2$ generating efficiency to be LY > PHR > SPI, although, the difference is not very significant. Further

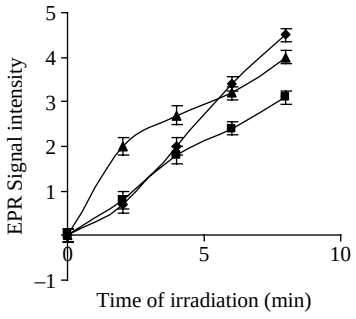


Figure 2. Plot showing comparison of EPR signal intensity for singlet oxygen generation by various phycocyanins vs time of irradiation.

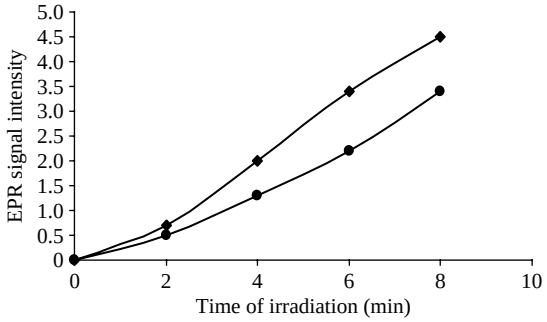


Figure 3. Quenching of photogenerated singlet oxygen generated by LY (◆) in the presences of 5 mM DABCO (●).

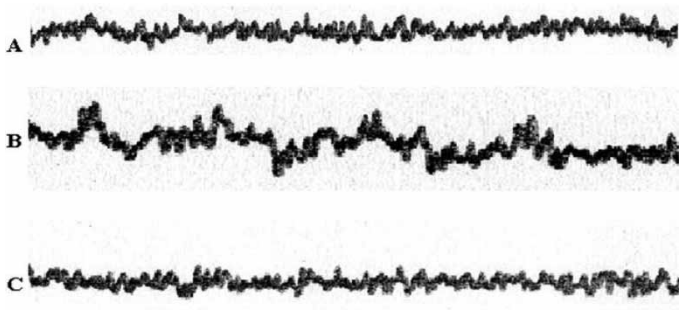


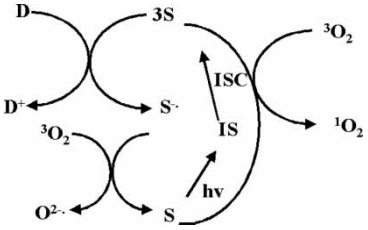
Figure 4. EPR spectra of DMPO adduct in aerated DMSO solution containing phycocyanin PHR (2 mg/ml) and DMPO (100 mM). (A) In the dark. (B) After 6-min irradiation (C) In the presence of SOD. Spectrometer setting: microwave power, 2 mW; modulation frequency, 100 kHz; modulation amplitude, 0.5; time constant, 0.1 s; scan rate, 4 min; scan width, 200 G; receiver gain, 500; line width, 1.1.

confirmation of the photo generation of the singlet oxygen was obtained by the widely used method of $^1O_2$ quenching by DABCO [20]. Figure 3 shows the reduction of the EPR signal intensity of TEMPOL in the presence of DABCO, confirming the formation of $^1O_2$.

Generation of superoxide anion

EPR- spin trapping technique was used for the detection of superoxide. Lifetime of the spin adduct DMPO- $O_2^{\cdot-}$ is short in protic solvent and the superoxide adduct decomposes to DMPO-OH [21]. Hence, EPR- spin trapping studies were carried out in DMSO in which the DMPO- $O_2^{\cdot-}$ adduct has a longer life time. No EPR signal was observed in dark as well as when DMPO alone was irradiated in DMSO. But irradiation of phycocyanin (2 mg/ml) with DMPO in aerated DMSO generated a 12-line EPR spectrum characteristic of the DMPO- $O_2^{\cdot-}$ adduct as shown in Figure 4. Other compounds also showed similar behavior. The pathways of singlet oxygen and superoxide generation are shown in Scheme 1. Addition of SOD (50 μg/ml) prior to irradiation prevents the formation adduct. The fact that the EPR signal intensity of this spin adduct is lower is in accordance with the possibility of the exited photosensitizer reacting with the sensitizer itself or with the solvent which in turn can cause the inhibition of the type I reaction [22].

In summary, we have demonstrated unambiguously the photogeneration of singlet oxygen by three phycocyanins using direct EPR-TEMPL method and the generation efficiency follows the order LY > PHR > SPI. Further based on the yield of $^1O_2$ and $O_2^{\cdot-}$ determined qualitatively by EPR, we conclude type II as the prominent mechanism of photosensitization by phycocyanins.



Scheme 1. Schematics of generation of $^1O_2$ and $O_2^{\cdot-}$.

Acknowledgements

We thank Prof P Sambasiva Rao of Chemistry Department, Pondicherry University, Pondicherry for EPR- measurements. We thank UGC for financial support under Centre for Potentials in Genomic Science, Madurai Kamaraj University, India. We also thank Ms/Strides Arcolab & CSIR for their financial support

References

[1] Boussiba S, Richmond AE. Isolation and characterization of phycocyanins from the blue-green alga *Spirulina platensis*. Arch Microbiol 1979;120:155–159.
[2] Marcos NC, Heary WL. US patent, 4, 886, 831 Che. Abstract. 1990 113,553j.
[3] Dougherty TJ, Gomer CJ, Henderson BW, Jori G, Kessel D, Korbelik M, Moan J, Peng Q. Photodynamic therapy. J Natil Cancer Inst 1998;90:889–905.
[4] Schopf JW. Precambrian micro-organisms and evolutionary events prior to the origin of vascular plants. P Biol Rev 1970;45:319–352.
[5] Patel Anamika, Sandhya Mishra, Ghosh PK. Kinetic studies on thermal denaturation of C-phycocyanin. Ind J Biochem Biophys 2004;41:254–257.
[6] Zhang S, Xie J, Zhang J, Zhao J, Jiang L. Electron spin resonance studies on photosensitized formation of hydroxyl radical by C-phycocyanin from *spirulina platensis*. Biochim et Biophys Acta 1999;1426:205–211.
[7] Jin-An He, Hu Y, Jiang L. Photodynamic action of phycobiliproteins: *In situ* generation of reactive oxygen species. Biochim et Biophys Acta 1997;1320:165–174.
[8] Kalayanaraman B, Felix CC, Sealy RC. Photoionization of melanine precursors: An electron spin resonance investigation using the spin trap 5,5-dimethyl-1-pyrroline-1-oxide (DMPO). Photochem Photobiol 1982;36:5–12.
[9] Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY. Generic assignments, strain histories and properties of the pure culture of cyanobacteria. J Gen Microbiol 1979;111:1–61.

[10] Chen F, Zang YM, Guo SY. Growth and phycocyanin formation in photohetrotropic culture. Biotechnol Lett 1996;18:603–608.
[11] Patel S, Mishra R, Pawar PK, Ghosh. Purification and characterization of C-phycocyanin from cyanobacterial species of marine and freshwater habitat. Protein Expr Purif 2005;40:248–255.
[12] Mothilal K, Johnson Inbaraj, Gandhidasan R, Murugesan R. Photosensitization with anthraquinone derivatives: Optical and EPR spin trapping studies of photogeneration of reactive oxygen species. J Photochem Photobiol Chem 2004; 162:9–16.
[13] Rajendran M, Ramasamy S, Rajamanicakam C, Gandhidasan R, Murugesan R. Photodynamic effects of two hydroxyanthraquinones. Biochim Biophys Acta 2003;1622(2):65–72.
[14] Krishna MC, Degraff W, Gonzalez TS, Amram Samuni FJ, Russo A, Mitchell JB. Mechanisms of hypoxic and aerobic cytotoxicity of mitomycin C in Chinese hamster V79 cells. Cancer Res 1991;51:6622–6628.
[15] Hahn SM, Wilson L, Krishna MC, Liebmann J, Degraff W, Gamson J, Samuni A, Venzon D, Mitchell JB. Identification of nitro-oxide as radioprotectors. Radiant Res 1992;132:87.
[16] Diwu ZJ, Lown JW. Photosensitization with anticancer agents: 15. Perylenequinonoid pigments as potential photodynamic therapeutic agents: Formation of semiquinone radicals and reactive oxygen species on illumination. Photochem Photobiol B 1993;18:131–143.
[17] Beuttner GR, Oberley LW. Considerations in the spin trapping of superoxide and hydroxyl radical in aqueous systems using 5,5-dimethyl-1-pyrroline-1-oxide. Biochem Biophys Res Commun 1977;83:69–74.
[18] Weishaupt KR, Gomer CJ, Doughertry TJ. Identification of singlet oxygen as the cytotoxic agent in photo-inactivation of a marine tumor. Cancer Res 1976;36:2326–2329.
[19] Buttner GR. Spin trapping: ESR parameters of spin adduts. Free Radic Biol Med 1987;3:259–303.
[20] Hongbo ana, Jie xie, Jingquan zhao, Zhanshuang li. Photogeneration of free radicals (1OHandHB12) and singletoxygen (1O2) by hypocrellin B in TX-100 micelles microsurroundings. Free Radic Res 2003;37:1107–1112.
[21] Finkelstein E, Rosen GM, Rauckman EJ. Production of hydroxyl radical by decomposition of superoxide spin- trapped adducts. Mol Pharmacol 1982;21:262–265.
[22] Su PingZhang, Jie Xie, Zhang Jian-ping, Zhao Jing-quan, Jiang Li-jin. Electron spin resonances studies on photosensitized formation of hydroxyl radical by C-Phycocyanin from *Spirulina platensis*. Biochem et Biophys Acta 1996;1426: 205–211.