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ORIGINAL PAPER

Purified C-phycocyanin from *Spirulina platensis* (Nordstedt) Geitler: a novel and potent agent against drug resistant bacteria

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Abstract The experimental data on the study of the antibacterial activity of purified phycocyanin, a protein-bound pigment isolated from blue-green alga, *Spirulina platensis* (Nordstedt) Geitler, Oscillatoriaceae are generalized and it was shown that phycocyanin was able to markedly inhibit the growth of drug resistant bacteria *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa and Staphylococcus aureus* while, no activity was recorded in *Acinetobacter baumanii* and *Enterococcus durans*, this is the first report of the activity of purified C-phycocyanin against drug resistant bacteria. The possible use of phycocyanin as a drug with associated antibacterial activity is discussed.

Keywords Drug resistant bacteria · MIC · Phycocyanin · *Spirulina platensis*

Introduction

Antibacterial research over the past 50 years has been focused on meeting medical needs caused by infectious, life-threatening pathogens. In spite of the introduction of a variety of antibacterial agents in multiple unrelated drug classes, resistance continues to emerge. Infections caused by antibiotic resistant bacteria represent a significant burden to healthcare systems worldwide today. The pharmaceutical industry must respond to these clinical challenges by bringing forward a stream of new agents with antibacterial activity against drug resistant bacteria (Bush 2004).

Spirulina platensis (*Arthrospira platensis* (Nordstedt) Gomont) (Nordstedt) Geitler, belonging to the Family Oscillatoriaceae the multicellular filamentous cyanobacterium comprises up to 40% of its total protein as major light-harvesting protein-pigment complexes as C-phycocyanin (C-PC) which have nutritional as well as medicinal properties (Zhang et al. 2004; Chen et al. 2003). Recent reports have credited C-phycocyanin with many pharmacological properties such as anti-inflammatory (Romay et al. 2003), antioxidant (Ge et al. 2006) antitumor activities (Li et al. 2005) radical scavenging properties and neuroprotective effects (Vadiraja and Madyastha 2000). Purified C-PC from *Spirulina* demonstrated its urokinase Plasminogen Activator (uPA) enhancing activity in human fibroblasts (Madhyastha et al. 2006). Our interest here is to demonstrate the in vitro activity of C-phycocyanin against drug resistant bacteria.

Materials and methods

Extraction, purification and characterization of C-phycocyanin

Phycocyanin was extracted from the *Spirulina platensis,* which were obtained from the Algal Culture Collection, Centre for Advanced Studies in Botany, University of Madras, Chennai, India. The micro alga *S. platensis* was cultured for 7–8 days in Zarrouk's medium in 4 L Erlenmeyer flasks, with photoperiod of 12 h light/dark provided by fluorescent lamps at a light intensity of 140 μmol photons m^{-2} s^{-1} and temperature of 30 ± 1°C. Experiments were initiated with 10% (v/v) of inoculum. Media agitation

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was carried out by bubbling air. For the preparation of crude C-phycocyanin, 1 g (ca.) of algal filaments were ground in 20 mL of 10 mM potassium phosphate buffer pH 7.0 and centrifuged at 10,000*g* for 30 min, and a C-phycocyanin containing clear blue supernatant was collected. It was fractioned with ammonium sulfate and precipitate was dissolved in a known volume of sodium phosphate buffer pH 7.0 and dialyzed against the same buffer. The dialyzed sample was chromatographed on a DE-52 anion exchange column and eluted with NaCl solution (0–0.5 M, 100 mL) at 1 mL/min. All visible and UV spectra of C-phycocyanin samples were measured on a Shimadzu 2001 spectrophotometer. The purity was evaluated according to the absorbance ratio A_{620}/A_{280} (Boussiba and Richmond 1979).

SDS–PAGE analysis was carried out according to the method of Laemmli (1970). Molecular weight markers were run simultaneously with the samples. To check molecular weight and intactness of the protein polyacrylamide gel under non-denaturing conditions was done. The protein samples were dissolved in sample buffer containing 10% sucrose and 0.003% bromophenol blue in Tris–HCl buffer, pH 6.8 and loaded onto the staking gel. The proteins were separated using 60 V at 4°C. Resolved proteins were transferred to nitrocellulose membranes. After 1 h of incubation with the blocking solution containing 5% low-fat milk in TBS (20 mM Tris–HCl, pH 7.5 and 0.9% NaCl), the membranes were washed twice with TBS-T (TBS containing 0.05% Tween 20). After being washed with in TBS-T, the blots were incubated with the primary antibody (anti C-phycocyanin) at a dilution of 1:1,000 in blocking solution for 1 h at room temperature. After being washed in TBS-T, the blots were incubated with horseradish peroxidase conjugated anti-rabbit IgG (1:20,000 for 1 h), washed for three times, and the proteins were detected using enhanced chemiluminiscence system (Amersham).

Bacterial strains

The drug resistant bacterial test organisms used in this present study obtained from Institute of Microbial Technology, Chandigarh, India, include Gram negative strains namely *Acinetobacter baumanii* Carbofuran resistance clinical strain, *Escherichia coli* (ATCC 25922) ciprofloxacin resistance, *Klebsiella pneumoniae* (ESBL-KP) ATCC 700603 and *Pseudomonas aeruginosa* MTCC 1034 and Gram positive strains viz., *Enterococcus durans* (P502) vancomycin resistant clinical strain and *Staphylococcus aureus* ATCC 25923 (MRSA).

Antibacterial assay

Antibacterial assay was carried out according to Bauer et al. (1966). Overnight grown culture of the pathogen with the turbidity of the culture adjusted to an OD of McFarland 0.5 at 580 nm was used for the assay. 0.1 mL of the precultured bacterial suspension was swabbed on Mueller–Hinton Agar plates. C-phycocyanin (100 µg) mixed with sterile distilled water and loaded onto 6.0 mm diameter sterile paper discs (Himedia) and dried in sterile chamber. Then these discs were impregnated on the assay plates and incubated at room temperature. Inhibition zones were recorded after 24 h. Ampicillin was used as positive control. Triplicates were maintained in the present study.

MIC

Minimum Inhibitory Concentration was determined using broth microdilution method (NCCLS 2003; Mazzanti et al. 2000; Devienne and Raddi 2002). All tests were performed in Mueller–Hinton agar broth (Himedia). Serial doubling dilution were prepared with a solution of maximum active seaweed extracts: Dimethylsulfoxide 95:5 in a 96-well microtiter plate over the range of 7–3,125 µg/mL. Overnight broth cultures of each strain were prepared and the final concentration of the microbe in each well was adjusted to 2×10^3 cfu/mL. Plates were incubated at 37°C for 24 h. The MIC was determined by reading the absorbance of each well using an automatic ELISA tray reader adjusted at 630 nm (SLT Spectra). The samples were analyzed in duplicate and the assay was repeated twice. The wells showing complete absence of growth were identified and 10 µL of each well were transferred to Mueller–Hinton agar plates and incubated at previously mentioned times and temperatures. Values are expressed as mean ± standard error and statistical significance was set at $P < 0.05$.

Results

In this study, 30–50% concentrations were used and the precipitate obtained from each step was identified using absorption spectral scanning. The 30% (w/v) ammonium sulphate solution precipitated other proteins whereas the 50% solution precipitated C-phycocyanin. The crude phycocyanin fractions obtained were respectively chromatographed on a DE 52 column and the C-phycocyanin containing sample was eluted with NaCl solution (0–0.5 M, 100 mL) at 1 mL/min^{-1}. The % yield of Phycocyanin with respect to the total protein obtained from the 50% Ammonium Sulphate fraction and the DE52 fraction are given in Table 1. The absorbance ratio of the C-phycocyanin fraction (A_{620}/A_{280}) was 4.69. Figures 1 and 2 depict the polyacrylamide gel electrophorograms of C-phycocyanin under non-denaturing and denaturing conditions. The former reveals a 39 kDa intact band and the later shows two bands of 20 and 19 kDa. Figure 3 shows

Table 1 Data of phycocyanin separation and purification from *Spirulina platensis*

Purification step	Total protein (mg)	Total C-PC (mg)	% C-PC from total protein	Absorbance ratios (A_{620}/A_{280})	Yield
Cell extract	280.00	128.00	45.71	1.20	100.00
Precipitations with 50% $(NH4)_2SO_4$	115.00	90.00	78.33	3.52	70.30
DE 52 fractions	62.00	59.74	96.35	4.69	46.67

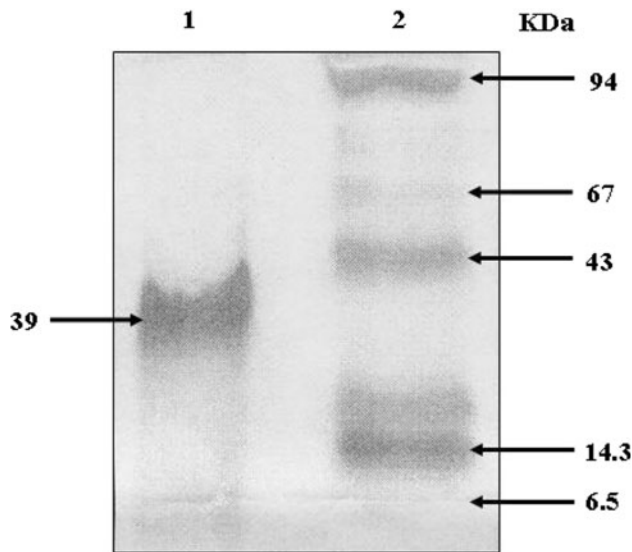


Fig. 1 Native PAGE (15%) of C-phycocyanin. *Lane 1* purified C-phycocyanin. *Lane 2* protein marker

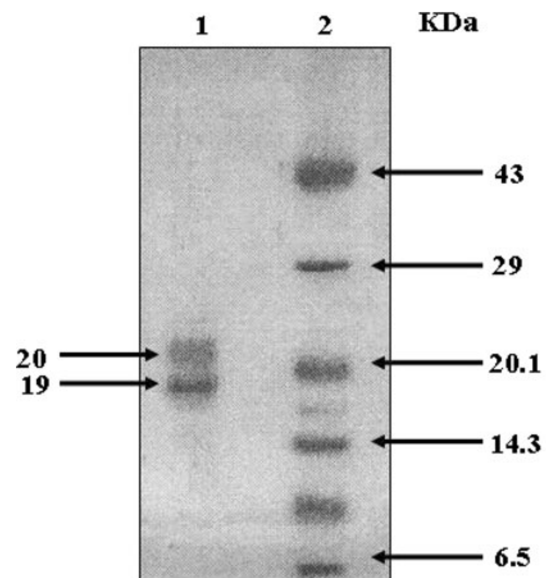


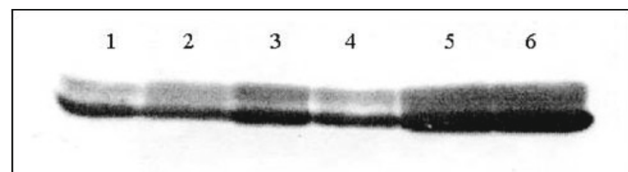
Fig. 2 SDS–PAGE (15%) of C-phycocyanin. *Lane 1* purified C-phycocyanin. *Lane 2* protein marker

the immunoblot for C-phycocyanin at different levels of purification.

Table 2 shows the activity of C-phycocyanin against antibiotic resistant bacteria. Activities were recorded against the Gram negative bacteria namely, *Pseudomonas aeruginosa* (18.00 ± 0.59), *Klebsiella pneumoniae* (16.00 ± 0.58), *Escherichia coli* (13.33 ± 0.67) and a Gram positive bacterium *Staphylococcus aureus* (9.33 ± 0.33). No activity was recorded against *Acinetobacter baumanii* and *Enterococcus durans*. The values of MIC are given in Table 3; *Pseudomonas aeruginosa* recorded the least value while the highest value was recorded for *Staphylococcus aureus*.

Discussion

The main objective of this study was to evaluate the applicability of C-phycocyanin purified from *Spirulina platensis* in the inhibition of growth of drug resistant bacteria. The blue pigment apart from being used as food



Lane 1 : Crude

Lane 2 : Pellet (8000 rpm)

Lane 3 : Supernatant (8000 rpm)

Lane 4 : 0-30% $(NH4)_2$ SO_4 supernatant

Lane 5 : 30-50% $(NH4)_2$ SO_4 pellet

Lane 6 : DE-52 fraction

Fig. 3 Western blot analysis of C-phycocyanin. *Lane 1*: crude. *Lane 2*: pellet (8,000 rpm). *Lane 3*: supernatant (8,000 rpm). *Lane 4*: 0–30% $(NH4)_2$ SO_4 supernatant. *Lane 5*: 30–50% $(NH4)_2SO_4$ pellet. *Lane 6*: DE-52 fraction

Table 2 Activity of C-phycocyanin against drug resistant bacteria

Pathogens	Zone of inhibition (mm in diameter)[a]
Acinetobacter baumanii	0.00 ± 0.00
Enterococcus durans	0.00 ± 0.00
Escherichia coli	13.33 ± 0.67
Klebsiella pneumoniae	16.00 ± 0.58
Pseudomonas aeruginosa	18.00 ± 0.58
Staphylococcus aureus	9.33 ± 0.33

[a] 100 µg per disc

Mean ± Standard error; Standard deviation $P \leq 0.05$

Table 3 MIC of C-phycocyanin

Pathogens	MIC (µg/ml)[a]
Escherichia coli	100
Klebsiella pneumoniae	75
Pseudomonas aeruginosa	50
Staphylococcus aureus	125

MIC minimum inhibitory concentration

[a] Values are the mean of 3 replicates using 1×10^3 cells of each culture

colorant has anticancer, antioxidant, antiviral and anti-inflammatory activities (Romay et al. 1998; Gonzalez et al. 1999; Hirata et al. 2000; Mathew et al. 1995). Also, phycocyanin is a powerful tonic agent for the immune system in animals and human, which providing protection from variety of diseases (Liu et al. 2000). Studies show *Spirulina's* antiviral activity in a number of in vitro models and in animals (Gustafson et al. 1989; Hayashi et al. 1996). *Spirulina* may be of benefit to HIV+ individuals not only because of the algae's action upon the virus itself, but also due to *Spirulina*'s effect upon on other viral or bacterial infections to which people with HIV/AIDS may be more susceptible.

The methanol extracts of *Spirulina platensis* in vitro have shown antimicrobial activity against four Gram-positive and six Gram-negative bacteria (Ozdemir et al. 2004). In the present study C-phycocyanin isolated from *Spirulina platensis* was able to control the growth of three species of Gram negative bacteria, namely, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* and a Gram positive bacterium *Staphylococcus aureus*.

To our knowledge this is the first report of the activity of purified C-phycocyanin against drug resistant bacteria. Having knowledge of impact of infectious diseases on global health and the continued emergence of antibiotic resistance bacteria, the study would help the biopharmaceutical industry in the timely and efficient development of new agents.

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