Cysteine rich cyanopeptide β2 from *Spirulina fusiformis* exhibits plasmid DNA pBR322 scission prevention and cellular antioxidant activity

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Isolation of three different active peptides from C-phycocyanin (C-pc) β chain of *S. fusiformis* and their biological properties are reported. Phycocyanin peptide β fraction 2 (cyanopeptide β2) facilitated both antioxidant and plasmid DNA strand scission prevention activity due to higher cysteine moieties in the isolated peptide. The peptide significantly scavenged the free radicals like 1,1-diphenyl-2-picryl hydrazyl and ferric reducing ability of plasma, increased the absorbance values in reducing power and also showed the higher trolox equivalent antioxidant capacity values in total reactive antioxidant potentials assay. Cyanopeptide β 2 also inhibited reactive oxygen species induced DNA pBR322 damage in dose dependent manner along with free radical scavenging properties suggesting the role in the DNA integrity which is also evident by DNA binding activity of peptide. In addition, the generation of reactive oxygen species (ROS) was dose dependent (10 and 20 ng/ml) and significantly quenched by cyanopeptide β2 in human fibroblast cell line TIG 3-20. *In vitro* cell scratch injury assay demonstrated the capacity of cyanopeptide β2 in cell migration in to wounded area suggesting fibroblast proliferation and migration. The results suggest that cyanopeptide β2 can be a free radical scavenger and effective peptide for future biomedical applications like wound healing, athrosclerosis, cell redox potential and hypoxia.

**Keywords:** Antioxidation, C-phycocyanin β peptide, DNA scission activity, *Spirulina fusiformis*

The free radicals and reactive oxygen species (ROS) can be effectively eliminated by an enzyme and non-enzymatic factors like glutathione system, ascorbic acid, polysaccharides and protein. Cyanobacteria have received great attention from researchers in recent years. Some cyanobacteria like *Spirulina* contain high amount of essential proteins, vitamins, minerals and many polysaccharides1. Among various metabolites of *Spirulina*, blue colored fluorescent protein C-phycocyanin (C-pc) gained immense application in biomedical and pharmacological fields2,3. However, little information on algal peptide application in medical field is available4. The peptides present in dietary proteins once liberated in the digestive tract by proteolytic enzymes, carry out a wide range of biological functions like antimicrobial, immunostimulatory and hypocholesterolemic activity5.

Usefulness of blue light in enrichment of C-pc during *Spirulina* culture6 and higher radical scavenging phenomenon by C-pc7 have been demonstrated. However, no information is available on the effects of C-pc peptide on cellular redox system and DNA damage. Hence, the present study has been undertaken to separate peptide fraction from C-pc β chain and to evaluate *in vitro* anti-oxidation reaction and DNA scission prevention with an aim to judge the biomedical properties of C-pc. In addition, the protective effect of the purified peptide against oxidation induced reactive oxygen scavenger (ROS) and prevention of cell injury in human TIG 3-20 was also determined.

### Materials and Methods

**Reagents**—Ferric chloride (FeCl₃), ethylenediamine tetra acetic acid (EDTA) potassium ferri cyanide [K₃Fe(CN)₆] and H₂O₂, were purchased from S.D. Fine Chem. Ltd (Mumbai, India). 1-diphenyl-2-picrylhydrazyl (DPPH), Tris-HCl, acetonitrile, trifluoro acetic acid (TFA), minimum essential medium (MEM), Fetal bovine serum (FBS) were from Sigma Chemical Co. (St. Louis, MO, USA). All reagents used were of analytical grade. C-phycocyanin was isolated to their pure forms (> 98%)
as per Madhyastha et al. Purity ratio (PR) = Absorbance 620/Absorbance 280:3.8

**HPLC condition**—The dialyzed C-pc solution was recovered by freeze drying and submitted to chromatographic separation methods. A cation-exchange CM-Sepharose column was employed for separation of α and β chains from the peptide-rich crude C-pc extract. The column (1.5×50 cm) was equilibrated and initially eluted with 0.02 M phosphate buffer, (pH 8.0). Elution of the bound fraction was carried out in a stepwise manner using NaCl from 0 to 1 M in the equilibration buffer. The two fractions were collected and diluted in a solution containing 0.065% TFA + 2% (v/v) acetonitrile and injected onto an HPLC C2/C18 ST 4.6/100 reverse-phase (RP) column (Shimadzu, Japan, model # LC 10, AD VP). The chromatography was developed at a flow rate of 0.5 mL min⁻¹ with 100% solvent A (0.065% TFA + 2% acetonitrile) for 10 min, 100% solvent B (80% acetonitrile containing 0.05% TFA) over 50 min and finally 100% solvent B over 10 min. Peptide fractions were monitored by on-line measurement of the absorbance at 280 nm. Amino acid sequencing of each fraction was performed by Edmens degradation method.

**Trypsin digestion of C-phyocyanin β chain**—β fraction (10 μL) of C-pc (20 μg/μL) was mixed with 15 μL of digestion buffer (50mM NH₄HCO₃) and 2.0 μL of reducing buffer (100 mM DTT) in a micro-centrifuge tube. The sample was incubated at 95°C and allowed to cool. Alkylation buffer (3 μL; Idoacemamide 100 mM) was added and incubated in dark at room temperature for 20 min. After reduction and alkylation process, 1 μL of trypsin (0.1 μg/μL) was added and incubated overnight at 37°C.

**Circular dichroism spectroscopy (C D Spectroscopy)**—Cyanopeptide β2 folding was examined by UV Vis circular dichroism spectroscopy (Jasco J-715 CD spectrophotometer, Tokyo, Japan) using quartz cuvettes with an optical path length of 1 mm. Initially the blank spectra (250–700 nm) of the buffer were recorded. The measurements were made using peptide concentration of 50 μM, and the peptide was added to sodium phosphate buffer directly before starting the measurement. Acid inactivated peptide (in 10 mM sodium phosphate buffer, pH 2.0) was used as negative control. All spectra were recorded at 25°C. The spectra were measured in the wavelength range from 250–700 nm with a 0.2 nm step resolution and a 1 nm bandwidth. The scanning rate was 50 nm/min with 2 sec response time. The signal-to-noise ratio was calibrated before scans. Data processing was carried out using the J-700 software package.

**Anti-oxidation assay**—1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay was conducted according to method of Blois. In short, DPPH was dissolved in ethanol to get final concentration of 500 μM. DPPH (0.5 ml) was mixed with 1.0 ml of 100 mM tris-HCl (pH 7.5) containing different concentrations of test samples in scavenging tubes. The tubes were incubated in dark for 30 min at room temperature and absorbance was recorded in a UV-Vis spectrophotometer at 517 nm. The antioxidant capacity was calculated as (%) of DPPH scavenging activity as per the formula (O.D of control – O.D of test sample ÷ O.D of control) × 100.

**Oxygen radical absorbance capacity (ORAC) assay**—ORAC-fluorescein assay kit (Oxford Biomedical Research Oxford, MI, USA) was used. Trolox standards (10-200 μM), fluorescein (10 μM), and AAPH (70 mM) solutions were prepared prior to use in phosphate buffer (75 mM, pH 7.4). C-pc (10 μl) and ascorbic acid was prepared in chloroform, acetone and trolox in phosphate buffer. Blank solutions in triplicate were also prepared using corresponding solvents to serve as controls. Different concentrations of C-pc with controls were placed in the well of a Nunc assay plate (Nalge Nunc International, NY, USA) and fluorescein (100 μl) was added to each well. The total volume in each well was made up to 250 μl using buffer solution. The plate was incubated in the preheated (37°C) microplate reader for 10 min under slow shaking condition. Subsequently, 20 μl of 2,2′-azobis(2-amidino-propane) dihydrochloride (AAPH) was added. The plate was read at excitation and emission wavelengths of 485 and 535 nm respectively, at 37°C at 3 min intervals for 60 min. Standard curve was obtained by plotting trolox concentrations against the average net area under the curve (AUC) values of the three measurements for each concentration. The net AUC values corresponding to a sample was calculated by subtracting the AUC values of the blank. A standard curve was generated from the net AUC of the trolox standards and used to assign trolox equivalence values to the test samples. Final ORAC values were calculated using the regression equation between trolox concentration and the net AUC and are expressed as μmol of trolox equivalents.
Ferric reducing ability of plasma (FRAP) assay—The ferric reducing ability of each standard solution and test compound was measured according to a modified protocol developed by Antolovich et al. A mixture of 0.1 M acetate buffer (pH 3.6), 10 mM ferric tripyridyl triazine (TPTZ), and 20 mM ferric chloride (10:1:1, v/v/v) was used to prepare the FRAP reagent. Readings at the absorption maximum (593 nm) were taken every 15 sec using a BIO-RAD spectrophotometer (BIO-RAD Instruments, USA), and the reaction was monitored for up to 10 min. Trolox solution was used to perform the calibration curves.

Reducing power assay—The reducing power of ascorbic acid, and test samples was determined according to the method of Oyaizu. Briefly, test compound (1.0 mg) in 2 mL of ethanol (96%) was mixed with phosphate buffer (3.0 mL, 0.2 M, pH 6.6) and potassium ferricyanide \([K_3Fe(CN)_6]\) (4.0 ml 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of Tri chloro acetic acid (TCA) (10% w/v) was added and the mixture centrifuged at 750 g for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and FeCl₃ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. All data are average of triplicate analyses.

Total reactive antioxidation potential (TRAP) assay—TRAP was determined according to the method of Ghiselli et al. based on the protection provided by antioxidants during a controlled peroxidation reaction. Briefly, 120 µl of diluted sample was added to 2.4 mL of phosphate buffer (pH 7.4), 375 µl distilled water, 30 µl diluted R-phycocerythrin and 75 µl azo-bis-2-amidinopropane (ABAP), the reaction kinetics were recorded at 38°C for 45 min (or more, if necessary) by a LS-55 luminescence spectrometer (Perkin-Elmer, Wellesley, MA). TRAP values were calculated from the length of the lag-phase due to the sample compared with that of Trolox.

DNA scission prevention assay—Efficacy of pBR322 plasmid DNA was determined as per Maxam and Gilbert. Briefly, 1 µg DNA pBR322 (Sigma Aldrich, St Louis, USA) was incubated with 3 mM H₂O₂ and subjected to UV radiation for DNA scission in presence of different concentrations (0, 2, 5, 10 and 20 ng/ml) of Cyanopeptide β2 at room temperature. After irradiation, sample was removed and loaded to 1% agarose gel and analysed by electrophoresis. Untreated pBR322 was run as a control. The DNA bands were visualised and analysed by gel documentation system (Sony, Corp, Japan).

DNAse 1 protection assay—This assay is used to know the plasmid DNA-peptide binding efficiency. Binding assay was performed according to standard protocol. The assays were performed by mixing 300 µg of the plasmid DNA (pBR322) with three different peptides (Cyanopeptide β 1, 2, 3 with or without H₂O₂) in 45 ml of HBS (Hepes-NaOH buffer containing 135 mM NaCl and 0.5 mM Na₂HPO₄, pH 7.2). After 30 min at room temperature, 5 ml of 20 mM MgCl₂ and 20 mM CaCl₂ were added followed by 5 ml of 0.5 mg/ml DNase I (Worthington, DPFF grade). After 30 min at 42°C, 50 ml of a stop solution consisting of 4 M ammonium acetate, 20 mM EDTA, and 2 mg/ml glycogen was added, and the reaction mixture was placed on ice. To dissociate the plasmid DNA from the peptide, 15 ml of 1% sodium dodecyl sulfate (SDS) was added prior to extraction with TE-saturated phenol/chloroform, followed by ethanol precipitation. The final pellet was re-suspended in 25 ml of dye mixture (TBE, 0.02% bromophenol blue, and 5% glycerol). Tris/borate/EDTA (TBE) consists of 90 mM Tris, 90 mM boric acid, and 2 mM EDTA, pH 8.0. An aliquot of 5.0 ml was applied to 1% agarose gel electrophoresis.

Human fibroblast cell (TIG 320) cell injury assay—Human lung fibroblast cell line, TIG 3-20 (JCRB0511) was purchased from the science research resource bank (HSRRB, Osaka, Japan). Fibroblast cells were cultured in MEM containing 10% heat-inactivated FBS, 100 units/ml of penicillin, and 100 µg/ml of streptomycin at 37°C in 5% CO₂. Standard cell scratch assay was performed to identify the recovery of cell injury by cyanopeptide β2. Briefly, cells were grown in 6 well plate (Greiner Bio-one, Cell Star, Germany) at density of 2×10⁵ per well. After sub confluence a small scratch was made with sterile cell scrapper (Nalge Nunc International, NY, USA) and injury border was marked on the plate. After repeated washing with phosphate buffer saline (PBS) cells were grown in presence or absence of cyanopeptide β2 (10 ng/ml) for 8 days experimental period. At 2, 4 and 8 days periods, images of the cells were obtained using digital photomicrography system (Nikon, Tokyo, Japan) and extend of injury recovery was analysed by image analysis software (Image J, NIH, Bethesda, USA).
**Intracellular reactive oxygen scavenger (ROS) assay**—Production ROS was monitored spectrofluorometrically by the 2′ 7′ dichloro fluorescein diacetate (DCFH-DA) method. DCFH-DA diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to the non-fluorescent DCFH, which can be rapidly oxidized to the highly fluorescent DCF in the presence of ROS. TIG 320 fibroblast cells were grown in 6 well plate at concentration of 2×10^5 per well up to sub confluent stage. After incubation with different concentrations of cyanopeptide β2 (0, 2, 5, 10 and 20 ng/ml) for 30 min, the cells were treated with H₂O₂ (10 μM) for 4 h. DCFH-DA was added to the culture plates at a final concentration of 5 μM and incubated for 4 h at 37°C in darkness. DCF fluorescence intensity was detected with emission wavelength at 530 nm and excitation wavelength at 485 nm using JASCO (FP-6200) spectrofluorometer (Tokyo, Japan).

**Statistical analysis**—All experiments were carried out in triplicate. Statistical analysis was performed in one way ANOVA and the significant differences was set at P<0.05.

**Results and Discussion**

Isolation and evaluation of medicinal properties of cyanopeptide β2 from cyanobacteria *Spirulina assuitensis* have been reported. Initially, α and β chains of pure C-pc were separated by HPLC method. Two distinct peaks were noticed at retention times of 15 and 19 min respectively (Fig. 1a). N-terminal analysis of α sub unit revealed the following sequence—MKTPLTEAVSIADSDQGRFLSSTQIQV LFGRFRQAKAGL and that of β sub unit showed the sequence—MFDAFTLVVSQADTRGEMLSTAQID ALSQMVAESNKYL. These results are confirmatory to our earlier studies. Further, active peptides of β chain were isolated by trypsin digestion and successive HPLC separation. Absorption pattern of different peptide fractions measured at the stop flow during the HPLC analysis with a distinct absorbance of 214 nm is given in Fig. 1b. The fractions 1, 2 and 3 were designated as cyanopeptide β1, cyanopeptide β2 and cyanopeptide β3 respectively. Analysis of the purity of each peptide fraction (1, 2 and 3) demonstrated the presence of three main bands in SDS-Tricine gel electrophoresis under reducing conditions (results not shown). Amino acid sequences of all three peptides revealed that the presence of cysteine repeats only in cyanopeptide β2 (Table 1).

Once the purity of all three peptides of C-pc β peptides chains was confirmed, different, in vitro antioxidant assays were performed. L-ascorbic acid was used as positive control in order to judge the bioactivity of each peptide. Initially, the IC₅₀ of each fraction was determined by doing regression analysis of different fractions at different concentrations in order to compare the activity. IC₅₀ values of all test compounds were significantly lower than that of...
standards like ascorbic acid (Table 2). Table 3 summarises the results of 5 different anti-oxidant assay systems namely DPPH, TRAP, reducing power, ORAC and FRAP. In the present study, five methods employed can be classified into different groups depending on the oxidizing reagents. DPPH and TRAP methods use radical and FRAP method use metal ion for oxidation. Highest and significant (P<0.05) activities of DPPH and TRAP activity in cyanopeptide β2 may be due to presence of cysteine repeats in peptide (Table 1). Significance of cysteine rich peptides like tracheal antimicrobial peptide (TAP) and defensins as anti microbial agent has been discussed. However, this is the first study to report antioxidant properties of cysteine rich cyanopeptide from C-pc. Thus, overall DPPH radical scavenging effect of the peptide was to donate electron and could react with free radicals to convert them to stable products and finally terminating radical chain reaction. Reducing power assay may serve as a significant indicator during antioxidant action. Increased absorbance of the reaction mixture indicates higher reducing power. The observed higher reducing power of the cyanopeptide β2 could be due to the presence of higher cysteine residue. Based on its amino acid composition and bioactivity cyanopeptide β2 is strikingly similar to the other antimicrobial cysteine rich peptides like thionins of plant kingdoms. ORAC assay is an inhibition method wherein sample is added to free radical generating system and finally inhibition of free radical action is measured. FRAP assay measures the ferric reducing ability of a sample. It is entirely different from the ORAC because there is no free radical or oxidant applied in the assay. In the present study cumulative sum of both ORAC and FRAP assay systems demonstrated that cyanopeptide β2 is better antioxidant peptide than others and is stronger than ascorbic acid (Table 3). Once the antioxidant properties of three different cyanopeptides were evaluated, the further study was focused on cyanopeptide β2 because it showed highest radical scavenging properties. The antioxidative properties of peptides were highly influenced by molecular mass and structure. CD spectrum analysis was carried out and results are depicted in Fig. 2. CD spectroscopic techniques are sensitive valuable tool to identify the structural changes in proteins and peptides. To investigate the possible conformation changes, the

<table>
<thead>
<tr>
<th>Assay</th>
<th>L-ascorbic acid (µg/ml)</th>
<th>Cyanopeptide β1 (ng/ml)</th>
<th>Cyanopeptide β2 (ng/ml)</th>
<th>Cyanopeptide β3 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH</td>
<td>75.4 ±5.3</td>
<td>5.8 ±0.2</td>
<td>9.2 ±2.3</td>
<td>11.5 ±2.5</td>
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<tr>
<td>TRAP</td>
<td>56.3 ±2.4</td>
<td>13.9 ±4.6</td>
<td>8.4 ±1.4</td>
<td>15.4 ±2.4</td>
</tr>
<tr>
<td>ORAC</td>
<td>21.2 ± 3.6</td>
<td>2.4 ± 1.3</td>
<td>9.6 ± 1.2</td>
<td>8.9 ± 1.7</td>
</tr>
</tbody>
</table>

IC50 values were determined by regression analysis of different doses of test compounds.

<table>
<thead>
<tr>
<th>Test compounds and assay</th>
<th>DPPH (% of scavenging DPPH radicals)</th>
<th>TRAP (µM TEAC equivalent)</th>
<th>Reducing Power (absorbance)</th>
<th>ORAC (Trolox equivalent)</th>
<th>FRAP (Mmol/L ferrous ions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid†</td>
<td>85.2±3.9</td>
<td>15.73±2.6a</td>
<td>0.20±0.03a</td>
<td>5.1±1.1</td>
<td>0.82±0.4a</td>
</tr>
<tr>
<td>Cyanopeptide β1 (ng/ml)</td>
<td>21.5±4.03a</td>
<td>1.4±1.2a</td>
<td>0.5±0.01a</td>
<td>5.3±2.1</td>
<td>1.4±0.5a</td>
</tr>
<tr>
<td>Cyanopeptide β2 (ng/ml)</td>
<td>78.1±8.6b</td>
<td>21.8±4.5b</td>
<td>12.3±2.9b</td>
<td>9.2±0.7</td>
<td>4.2±3.2b</td>
</tr>
<tr>
<td>Cyanopeptide β3 (ng/ml)</td>
<td>31.9±3.5a</td>
<td>10.3±1.1</td>
<td>1.8±0.4a</td>
<td>3.7±1.9</td>
<td>1.8±0.3</td>
</tr>
</tbody>
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*IC50 values; † positive control

Values are significantly different (P<0.05) between different groups.
secondary structure of cyanopeptide β2 was determined by far UV CD spectra and compared with acidic form that is cyanopeptide β2 dissolved in 10 mM sodium phosphate buffer pH 4.0. In 10 mM sodium phosphate buffer and at pH 7.2, the CD spectrum of the cyanopeptide β2 was found to be more stable than the acid soluble form. This result suggested that cyanopeptide β2 maintained the secondary structure. By using K2D program, the α-helix content for cyanopeptide β2 was 35.2. In the present study we also validated antioxidant properties of cyanopeptide β2 in human cell culture models. The intracellular ROS level in fibroblast was evaluated by conversion reaction of DCFH-DA to DCF. Dose dependent reduction of cyanopeptide β2 on ROS level in H2O2 treated cells is shown in Fig. 3. A significant (P<0.5) increase of DCF fluorescence intensity caused by H2O2 dropped significantly as doses of cyanopeptide β2 increases. Oxidative susceptibility of a given amino acid in peptide also dictated in large part by its R group, nucleophilic sulfur, aromatic and imidazole containing side chain.23 The cysteine residue of the cyanopeptide β2 in the present study was expected to prevent lipid and other biomolecules by donating protons to peroxo radicals and other free radicals in the cell as evident from ROS scavenging properties.

Fig. 3—Effect of cyanopeptide β2 on H2O2 induced intracellular ROS level. TIG 320 cells were incubated with different concentrations of cyanopeptide β2 (0, 2, 5, 10 and 20 ng/ml) for 30 min, later treated with H2O2 (10 μM) for 4 h. DCFH fluorescence intensity was measured expressed as percentage of DCFH. * P<0.5

Fig. 4—Fibroblast cell injury assay. Sub confluent human fibroblast, TIG 320 cells were treated with or without cyanopeptide β2 (10 ng/ml). Wound edge is marked by black line. The rate of cell migration to wounded area was photographed for various time period of 0, 2, 4 and 8 days.
The observed protective effect of peptide can be explained by its ability to scavenge OH radical due to cysteine residues in peptide since H2O2 damages base pair of DNA and induces strand to break28.

To conclude, cysteine rich cyanopeptide β2 showed significant antioxidant potential in terms of scavenging free radicals, prevents fibroblast cell injury, protects DNA from H2O2. This could be employed as an adjuvant to the conventional nutraceutical for prevention of DNA damage and cell injury.

**References**


