Biochemical and Biophysical Research Communications **275,** 20–25 (2000)
doi:10.1006/bbrc.2000.3270, available online at http://www.idealibrary.com on IDEAL®

C-Phycocyanin: A Potent Peroxyl Radical Scavenger *in Vivo* and *in Vitro*

Vadiraja B. Bhat* and K. M. Madyastha†,[1]
†*Department of Organic Chemistry, Indian Institute of Science, Bangalore, 560 012, India; and* **Chemical Biology Unit, JNCASR, Bangalore, 560 064, India*

Received July 11, 2000

C-Phycocyanin (from *Spirulina platensis*) effectively inhibited CCl_4-induced lipid peroxidation in rat liver *in vivo*. Both native and reduced phycocyanin significantly inhibited peroxyl radical-induced lipid peroxidation in rat liver microsomes and the inhibition was concentration dependent with an IC_{50} of 11.35 and 12.7 μM, respectively. The radical scavenging property of phycocyanin was established by studying its reactivity with peroxyl and hydroxyl radicals and also by competition kinetics of crocin bleaching. These studies have demonstrated that phycocyanin is a potent peroxyl radical scavenger with an IC_{50} of 5.0 μM and the rate constant ratios obtained for phycocyanin and uric acid (a known peroxyl radical scavenger) were 1.54 and 3.5, respectively. These studies clearly suggest that the covalently linked chromophore, phycocyanobilin, is involved in the antioxidant and radical scavenging activity of phycocyanin.

***Key Words: Spirulina platensis;* C-phycocyanin; reduced phycocyanin; peroxyl radical scavenger; hepatotoxicity.**

The nutritional and therapeutic values of *Spirulina*, a blue green algae are very well documented (1–3). C-Phycocyanin, a water soluble protein pigment is one of the major constituents of *Spirulina platensis*. Its various medicinal as well as pharmacological properties have been reported earlier (4–7). We have demonstrated that C-phycocyanin significantly reduces carbon tetrachloride (CCl_4) and *R*-(+)-pulegone-induced hepatotoxicity in rats (7). The hepatoprotective effect of phycocyanin could be due to the inhibition of some of the cytochrome P450 mediated reactions involved in the formation of reactive metabolites or its ability to act as an efficient radical scavenger or both (7). In fact it has been shown that the hepatotoxic effects of CCl_4 are due to its metabolic activation in the liver endoplasmic reticulum to reactive metabolites such as haloalkane free radicals with toxic consequences (8). These free radicals interact with membrane lipids and initiate the chain reaction of lipid peroxidation leading to tissue damage (9). Membrane lipid peroxidation is in fact responsible for the leakage of cytosolic enzymes to serum during chemical-induced toxicity. Earlier we have demonstrated that phycocyanin completely inhibits the leakage of glutamate pyruvate transaminase (SGPT) to serum after a massive dose of CCl_4 (7). So it is reasonable to assume that phycocyanin efficiently scavenges free radicals, inhibits the membrane lipid peroxidation and protects the liver against chemical-induced damage. In fact recently the antioxidant and anti-inflammatory properties of phycocyanin have been reported (4, 5). In the present communication, we report the radical scavenging property of phycocyanin *in vivo* and *in vitro*. We have also demonstrated that phycocyanin with a reduced chromophore also inhibits radical induced lipid peroxidation. We also present evidence to support the involvement of chromophore in the radical scavenging property of phycocyanin.

MATERIALS AND METHODS

C-Phycocyanin (isolated from *Spirulina platensis*) was a generous gift from Cyanotec Bio-products (P) ltd. Bangalore, India. It was further purified as reported earlier (10). The fractions showing an absorbance ratio of 618 nm/280 nm greater than 4 were pooled. The purity of phycocyanin prepared was examined by native PAGE and SDS–PAGE. The pure phycocyanin was dialyzed extensively against water and then subjected to electrospray ionization mass spectrometry to determine the molecular mass. HP-1100 MSD mass spectrometer was used for this purpose.

The chromophore in phycocyanin was reduced using $NaBH_4$ and the reaction was monitored spectrophotometrically. Procedure followed was similar to that used for the reduction of biliverdin-protein complex using $NaBH_4$ (11). After the reaction, it was dialyzed against water and freeze-dried.

Chemicals. 2,2′-Azo-bis(2-amidinopropane) hydrochloride (AAPH) was obtained from Aldrich Chemical Co. (St. Louis, MO). Crocin was isolated from saffron by water/methanol extraction as reported earlier (12). The extract containing crocin was diluted with 10 mM phosphate

[1] To whom correspondence should be addressed. Fax: 091-080-3600529. E-mail: kmm@orgchem.iisc.ernet.in.

0006-291X/00 $35.00

buffer (pH 7.4), and estimated using extinction coefficient 89,000 M^{-1} cm^{-1} reported for crocin in aqueous solution (13). CCl_4-induced lipid peroxidation in rat liver *in vivo*.

The liver injury in rat was induced as reported earlier (7). Male albino rats weighing 160–180 g were used. All the treatments were carried out intraperitoneally (i.p.). Carbon tetrachloride (CCl_4) at a dosage of 0.6 ml/kg was administered as a suspension in coconut oil (0.3 ml). To find out the effect of phycocyanin on CCl_4-induced lipid peroxidation, rats were pretreated with phycocyanin (50–200 mg/kg body wt) dissolved in water (0.5 ml) 3 h prior to the administration of CCl_4 (0.6 ml/kg). Animals were sacrificed by cervical dislocation 24 h after CCl_4 administration. Two sets of control experiments were carried out: (i) rats receiving only the vehicle and (ii) rats receiving vehicle with phycocyanin. Both the control and experimental rats were housed separately in cages with free access to food and water.

The liver was removed after perfusion *in situ* with ice-cold KCl (1.15%) and 10% liver homogenate was prepared in ice-cold KCl (1.15%) solution. The extent of hepatic lipid peroxidation was assayed by measuring malondialdehyde (MDA) with thiobarbituric acid (TBA) as described earlier (14). 1,1,3,3-Tetraethoxypropane was used as standard MDA. The results are expressed as nanomoles of MDA/gram wet weight of liver.

Peroxyl radical-induced lipid peroxidation in rat liver microsomes. Microsomal fraction was prepared from the livers of male albino rats (160–180 g body wt) as previously described (15). The microsomal pellet was washed and suspended in 1.15% KCl solution. The protein was determined by the method of Lowry *et al.* (16).

Freshly prepared microsomes (1.5 mg) were preincubated in potassium phosphate buffer (10 mM, pH 7.4), with or with out phycocyanin (1–250 μM) in a total volume of 1.0 ml for 10 min at 37°C. Then the lipid peroxidation was initiated by the addition of AAPH (20 mM, final concentration) and incubated for a further period of 1 h at 37°C. The extent of lipid peroxidation was assayed by measuring thiobarbituric acid (TBA)-reactive substances (TBARS) in microsomal membranes as reported earlier (17) with minor modification. Briefly, 3 ml of stopper solution [TCA (15%, w/v)-TBA (0.375%, w/v)-HCl (0.125 M)-BHT (0.6 mM)] was added, mixed and centrifuged at 10,000*g* for 15 min at 4°C. Phycocyanin reacts with TBA at higher temperature yielding a pink chromogen with an absorption peak at 528 nm. So to prevent this reaction, phycocyanin was removed by centrifugation after addition of stopper solution. The supernatant was transferred, boiled for 30 min, cooled and used to quantify TBARS photometrically at 532 nm. The results are expressed as % inhibition, which represents the degree of protection by phycocyanin against the AAPH-induced membrane lipid peroxidation.

Interaction of phycocyanin with peroxyl and hydroxyl radicals. In these studies, AAPH and Fenton reagent were used for the generation of peroxyl and hydroxyl radicals, respectively. Phycocyanin (10 μM) dissolved in 1.0 ml of phosphate buffer (10 mM, pH 7.4) was taken in a 1 ml quartz cuvette thermostated at 37°C. The reaction was initiated by adding freshly prepared AAPH solution (0.5 M in 20 μl) or a mixture of ferrous iron solution (20 μM), EDTA (100 μM), H_2O_2 (1.42 mM) and ascorbate (100 μM). While generating the hydroxyl radicals, ferrous iron solution and EDTA were added to phycocyanin and the reaction was initiated by the addition of a mixture of H_2O_2 and ascorbate. The final reaction mixture contained phycocyanin (10 μM), phosphate buffer (10 mM, pH 7.4) and AAPH (10 mM)/or Fenton reagent in a total volume of 1.0 ml. The changes in the UV–Vis spectra of phycocyanin were recorded between 300–700 nm for every 5 min in Shimadzu UV2100 thermostated spectrophotometer.

Crocin bleaching assay for peroxyl radical scavenging activity of C-phycocyanin. The reactivity of the phycocyanin with peroxyl radicals was measured by competition kinetics of crocin bleaching in the presence of peroxyl radicals generated by thermal decomposition of a azo compound (12). The test was carried out at 40°C in phosphate buffer (10 mM, pH 7.4) containing crocin (10 μM) and increasing concentrations (0–50 μM) of phycocyanin in a total volume of 1 ml. Uric acid (0–10 μM) was used as a known peroxyl radical scavenger. The peroxyl radicals were generated by adding AAPH (10 mM) and the rate of crocin bleaching was recorded at 440 nm in a thermostated spectrophotometer. The bleaching rate was linear 1.5 min after the addition of AAPH and the rate from 2 to 5 min was used for calculations. Bleaching rates were plotted as V_b/V_a versus [A]/[C], according to the equation $V_b/V_a = 1 + K_a/K_c$. [A]/[C], where V_b is the basal bleaching rate of crocin in the absence of phycocyanin, V_a is bleaching rate of crocin in the presence of phycocyanin, [C] and [A] are the concentrations of crocin and phycocyanin, respectively. K_a and K_c are the rate constants for the reaction of the peroxyl radical with phycocyanin and crocin, respectively. This plot gives a straight line, intersecting the ordinate, with a slope of K_a/K_c.

Measuring hydroxyl radical scavenging. Hydroxyl radical scavenging activity of phycocyanin was estimated by inhibition of deoxyribose degradation as described earlier (18).

RESULTS AND DISCUSSION

C-Phycocyanin used in the present study was homogeneous as judged by electrophoresis and absorption spectra ($A_{max}/A_{280} > 4.2$). Phycocyanin having an A_{618}/A_{280} above 4.0 was considered pure (19). The molecular mass of phycocyanin monomer as determined by electrospray ionization mass spectrometry (Fig. 1) was 37,468.5 mass units, 10.5 mass units less than the calculated mass of 37,479.0 for native phycocyanin. Amino acid sequence of α and β chains were retrieved from SWISS-PROT database (20) with primary accession #P72509 and P72508, respectively. The molecular mass of α and β subunits were 18,186.56 and 19,281.94 mass units, respectively (Fig. 1).

Exposure of animals to CCl_4 results in the cell damage and lipid peroxidation is generally invoked as an explanatory event that leads to injury (21). In the present study the effect of C-phycocyanin pretreatment on CCl_4-induced hepatic lipid peroxidation was studied and the results are presented in Fig. 2. Malondialdehyde (MDA) used as an index of hepatic lipid peroxidation. Consistent with the earlier report (20) that a single i.p. administration of CCl_4 (0.6 ml/kg) to rats caused marked increase in liver MDA level (Fig. 2). Phycocyanin when administered alone did not change the level of MDA and it was found to be similar to that of control value. However, administration of phycocyanin (50–200 mg/kg body wt) 3 h prior to CCl_4 treatment resulted in significantly lower production of MDA than found in rats receiving only CCl_4. The liver MDA level was nearly 5-fold greater in CCl_4 treated rats than control rats or rats treated with the combination of phycocyanin and CCl_4 (Fig. 2). It was also noticed that serum glutamate pyruvate transaminase (SGPT) levels in rats treated with the combination of phycocyanin (50–200 mg/kg) and CCl_4 (0.6 ml/kg) were similar to those of control values (results are not shown). It is known that in CCl_4 intoxication, free radicals arising from its biotransformation induce lipid peroxidation.

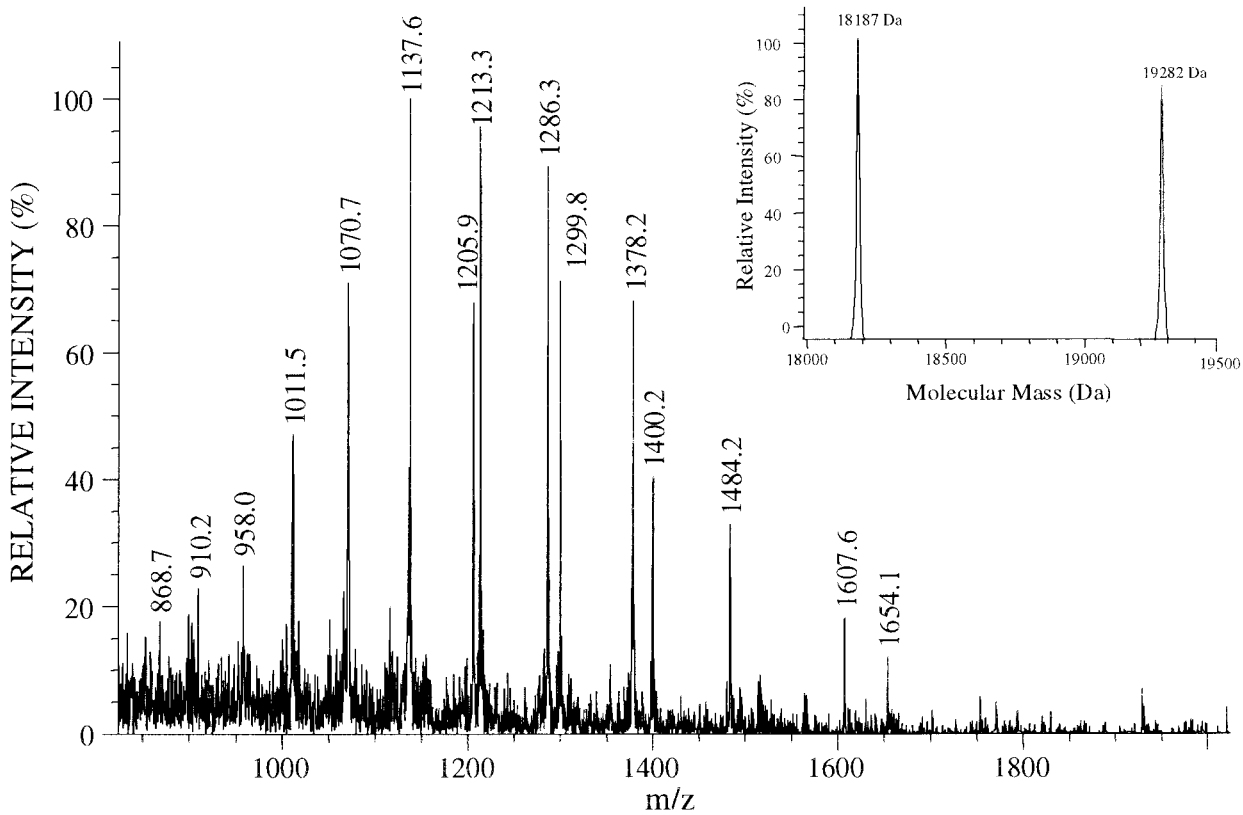

FIG. 1. Electrospray ionization-mass spectrum of C-phycocyanin monomer. The molecular mass of α and β subunit derived from the deconvoluted spectrum is shown in the inset.

The biotransformation is catalyzed by liver microsomal cytochrome P450 system (21–23). The trichlormethyl radical ($CCl_3^{\bullet}$) initially formed is relatively unreactive and this carbon centered radical readily reacts with O_2 to form a peroxyl radical ($CCl_3^{O\bullet}$) which is a good initiator of lipid peroxidation (9, 24). Earlier we have noticed that phycocyanin when administered alone to rats did not alter the liver function and the level of cytochrome P450 was similar to that of control value (7). This suggests that the protection by phycocyanin against CCl_4-induced lipid peroxidation may not be related to a less formation of reactive metabolites of CCl_4, but due to the unique ability of phycocyanin to scavenge reactive radicals.

The effect of phycocyanin on peroxyl radical-induced lipid peroxidation in rat liver microsomes was studied *in vitro* (Fig. 3). Hydrophilic radical initiator AAPH was used as a source of peroxyl radicals and the extent of membrane lipid peroxidation was measured by the formation of TBARS. In the present study it was noticed that phycocyanin inhibits the azo-initiated rat liver microsomal lipid peroxidation in a concentration dependent fashion with an IC_{50} value of 11.35 μM (Fig. 3). Earlier it was shown that phycocyanin inhibits the iron-ascorbate-induced rat liver microsomal lipid peroxidation with an IC_{50} of about 326.9 μM (12 mg/ml) (4). So it appears that phycocyanin scavenges peroxyl radicals more efficiently than hydroxyl radicals *in vitro*. Phycocyanin at 200 μM concentration inhibits nearly 95% of peroxyl radical-induced lipid peroxidation (Fig. 3).

Phycocyanin monomer contains two protein subunits (α and β) with three bilin chromophores, phycocyanobilin covalently attached to the apoprotein by thioether bonds to cysteine residues. Phycocyanobilin is an analogue of biliverdin and it has been reported earlier that addition of $NaBH_4$ rapidly reduces the biliverdin–albumin complex to bilirubin-albumin complex as detected by a color change (blue-green to yellow) and UV-visible spectroscopy (11). It has also been shown that during this facile transformation the C-10 methine bridge in the chromophore is reduced (11). Following this method we have reduced the chromophore in the native phycocyanin using solid $NaBH_4$. Reduction resulted in the color change (deep blue to greenish yellow) and disappearance of absorption at 360 and 618 nm and appearance of strong absorption near 418 nm in the UV-visible spectrum. Although reduced phycocyanin has not been characterized, the spectral characteristics resemble to that of phycocyanorubin, an analogue of bilirubin (25). This suggests that $NaBH_4$ possibly could have reduced the C-10 me-

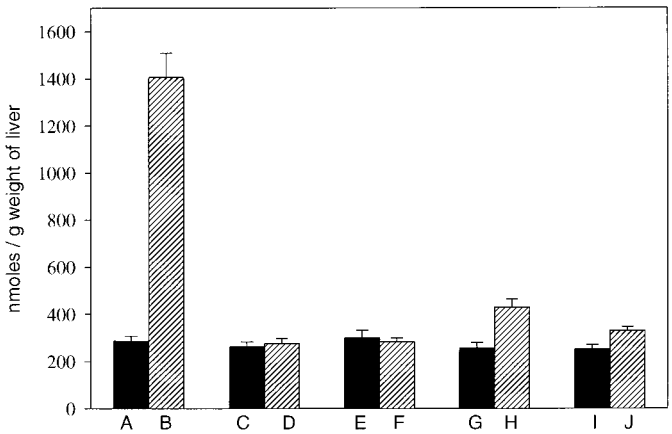

FIG. 2. Effect of C-phycocyanin on CCl_4-induced hepatic lipid peroxidation *in vivo* in rats. The extent of tissue lipid peroxidation was measured as MDA. Results were expressed as nanomoles of MDA/gram wet weight of liver. (A) Control, (B) CCl_4 (0.6 ml/kg), C, E, G, and I received 50, 100, 150, and 200 mg phycocyanin alone/kg, respectively; D, F, H, and J received 50, 100, 150, and 200 mg phycocyanin/kg, respectively, 3 h prior to CCl_4 (0.6 ml/kg) administration (i.p.). Values represent mean ± SD of 3 independent experiments, each consisting of tissues pooled from 4–6 rats.

thine bridge in the chromophore. It is interesting to note that the reduced (chemically modified) phycocyanin also efficiently inhibited peroxyl radical-induced lipid peroxidation in rat liver microsomes and the inhibition was dose dependent with an IC_{50} value 12.7 μM (Fig. 3). In fact both native and reduced phycocyanin inhibited lipid peroxidation almost to the same extent (Fig. 3). When reduced phycocyanin was used as an inhibitor of lipid peroxidation, it changes its color from greenish yellow to deep blue indicating that the

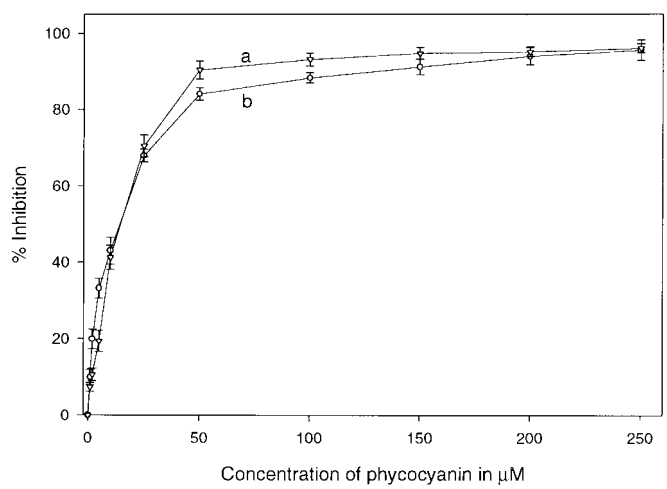

FIG. 3. The inhibitory effect of native phycocyanin and $NaBH_4$ reduced phycocyanin on peroxyl radical-induced lipid peroxidation (TBARS formation) in rat liver microsomes. The results are expressed as % inhibition of lipid peroxidation. The experimental details are as described under Materials and Methods.

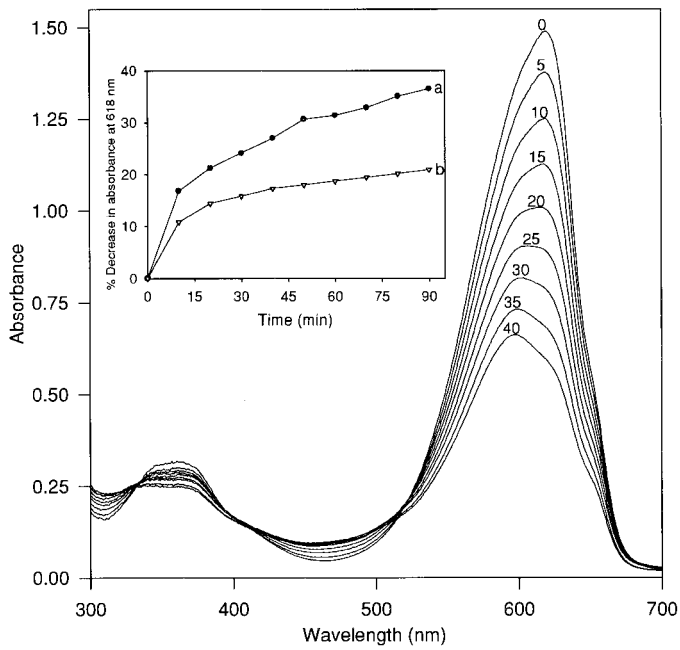

FIG. 4. Time-dependent spectral changes associated with the AAPH-induced oxidation of phycocyanin (10 μM) in phosphate buffer (10 mM, pH 7.4) at 37°C. The reaction was started by the addition of AAPH (10 mM) to the reaction mixture. Number indicate the time in minutes elapsed after the addition of AAPH. Inset: Time-dependent decrease in the absorbance at 618 nm associated with the hydroxyl radical induced oxidation of phycocyanin (10 μM) in phosphate buffer (10 mM, pH 7.4) at 37°C. (a) In the presence of EDTA and (b) in the absence of EDTA. The experimental details are as described under Materials and Methods.

chromophore gets re-oxidized to phycocyanobilin. This was further supported by our observation that when reduced phycocyanin was incubated with AAPH (10 mM) at 37°C, there was a rapid decrease in the absorption at 418 nm with the concomitant appearance of peaks at 618 and 360 nm in the UV-visible spectrum indicating that oxidation of phycocyanorubin to phycocyanobilin by peroxyl radical. In fact Fig. 4 clearly shows the transient formation of native phycocyanin during the interaction of peroxyl radical with reduced phycocyanin. Similar observations have been made earlier during the peroxyl radical mediated transformation of bilirubin to biliverdin (26). The aforementioned results clearly indicate that the chromophore (bilin) and not the apoprotein is directly involved in the antioxidant and radical scavenging properties of phycocyanin.

The involvement of chromophore, phycocyanobilin in the radical scavenging activity was established by studying the reactivity of phycocyanin with peroxyl radicals. Phycocyanin when incubated with AAPH (10 mM) at 37°C, there was a significant decrease in the absorption at 618 nm (60% decrease) and a shift in the absorption maxima at 618 nm (21.5 nm shift) toward lower wavelength was noticed (Fig. 5). The decrease in

the absorption is accompanied with disappearance of color. Similar spectral changes have also been noticed when phycocyanin was incubated with hydroxyl radical generating system (Fenton reagent) containing EDTA (Fig. 5). Under these conditions, there was a significant decrease in the absorption at 618 nm (36.5% decrease). However the decrease was less significant (20%) when incubation was carried out in the absence of EDTA (Fig. 5). This is possibly due to the fact that in the absence of EDTA, phycocyanin removes the free iron ion from the medium, which affects the hydroxyl radical formation, and in turn its interaction with phycocyanin. To confirm the iron binding property of phycocyanin, fluorescence quenching experiment was carried out in 50 mM sodium acetate buffer, pH 5.2 and it was found that phycocyanin interacts with iron ion with an association constant of $1.11 \pm 0.06 \times 10^5\ M^{-1}$.

The interaction of peroxyl radical with phycocyanin and its ability to scavenge peroxyl radical were further analyzed by the competition kinetics of crocin bleaching (Fig. 6). In these experiments peroxyl radicals were generated by the thermal decomposition of the azo compound AAPH. These studies demonstrated that phycocyanin is a potent peroxyl radical scavenger with an IC_{50} of 5.0 μM. Under these experimental conditions, uric acid, a known peroxyl radical scavenger had an IC_{50} of 1.9 μM. The rate constant ratios (K_{rel}) obtained for phycocyanin and uric acid were of 1.54 and 3.5, respectively (Fig. 6). The high rate constant for the interaction of phycocyanin with peroxyl radical suggests that the hepatoprotective effect of phycocyanin is due to its ability to scavenge reactive radicals.

Earlier it was shown that phycocyanin is a hydroxyl radical scavenger by deoxyribose degradation assay (4). Consistent with the earlier report (4), we have noticed that phycocyanin interacts with hydroxyl radical with a reaction rate constant (K_s) of $1.9 \times 10^{11}\ M^{-1}\ s^{-1}$ and inhibits the deoxyribose degradation with an IC_{50} value of 28 μM. When the deoxyribose assay was carried out in the absence of EDTA, phycocyanin was found to be a more potent inhibitor of deoxyribose degradation with an IC_{50} value of 13 μM. This suggest that phycocyanin chelate with iron ion and hence protect the target molecule, deoxyribose.

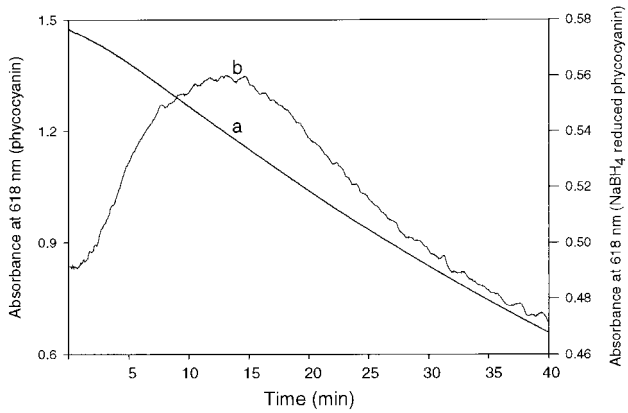

FIG. 5. Time-dependent changes in the absorbance at 618 nm associated with the peroxyl radical-induced oxidation of (a) native phycocyanin (10 μM) and (b) $NaBH_4$ reduced phycocyanin in phosphate buffer (10 mM, pH 7.4) at 37°C.

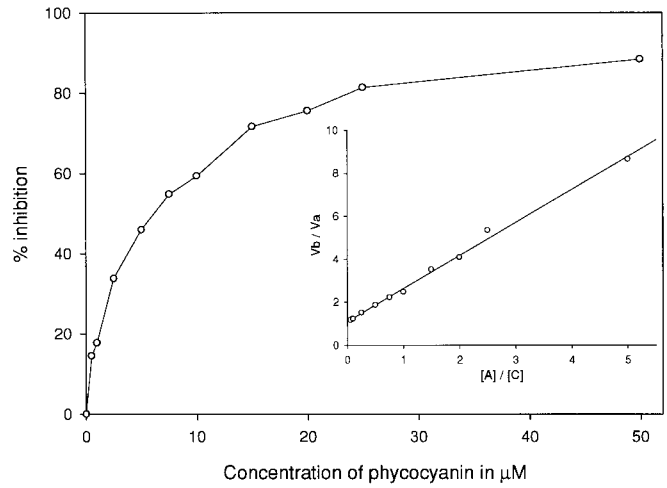

FIG. 6. Dose–inhibition curve of phycocyanin. The inhibitory effect of phycocyanin on peroxyl radical-induced bleaching of crocin in phosphate buffer (10 mM, pH 7.4) at 40°C. The experimental details are as described under Materials and Methods. Inset: competition kinetic plot of phycocyanin toward crocin in the AAPH-induced radical reaction. Phycocyanin concentration ranges from 0 to 50 μM. The slope of the straight line indicates the relative capacity of phycocyanin to interact with peroxyl radical according to the equation presented in the text.

In conclusion, the antioxidant property of phycocyanin was established on the basis of experiments carried out both in *vivo* and *in vitro.* The radical assisted bleaching of chromophore (bilin group) in phycocyanin clearly indicates its involvement in the scavenging of reactive oxygen radicals. The study provides an explanation for the anti-inflammatory property of phycocyanin.

ACKNOWLEDGMENTS

Financial assistance from CSIR (New Delhi, India) and JNCASR (Bangalore, India) is gratefully acknowledged.

REFERENCES

1. Bockow, B. I. (1998) United States Patent No. 05709855.
2. Kay, R. A. (1991) *Crit. Rev. Food Sci. Nutr.* **30,** 555–573.
3. Gonzalez De Rivera, C., Miranda-Zamora, R., Diaz-Zagoya, J. C., and Juarez-Oropeza, M. A. (1993) *Life Sci.* **53,** 57–61.
4. Romay, C., Armesto, J., Remirez, D., Gonzalez, R., Ledon, N., and Garcia, I. (1998) *Inflamm. Res.* **47,** 36–41.
5. Gonzalez, R., Rodriguez, S., Romay, C., Ancheta, O., Gonzalez, A., Armesto, J., Remirez, D., and Merino, N. (1999) *Pharmacol. Res.* **39,** 55–59.

6. Morcos, N. C., and Henry, W. L. (1989) United States Patent No. 04886831.
7. Vadiraja, B. B., Gaikwad, N. W., and Madyastha, K. M. (1998) *Biochem. Biophys. Res. Commun.* **249,** 428–431.
8. Boyd, M. R., Statham, C. N., and Longo, N. S. (1980) *J. Pharmacol. Exp. Ther.* **212,** 109–114.
9. Slater, T. F., Cheeseman, K. H., and Ingold, K. U. (1985) *Philos. Trans. R. Soc. London B* **311,** 633–645.
10. Boussiba, S., and Richmond, A. E. (1979) *Arch. Microbiol.* **120,** 155–159.
11. Trull, F. R., Ibars, O., and Lightner, D. A. Arch. (1992) *Biochem. Biophys.* **298,** 710–714.
12. Bors, W., Michel, C., and Saran, M., (1984) *Biochem. Biophys. Acta* **796,** 312–319.
13. Jorgensen, L. V., Andersen, H. J., and Skibsted, L. H. (1997) *Free Radical Res.* **27,** 73–87.
14. Ohkawa, H., Ohishi, N., and Yagi, K. (1979) *Anal. Biochem.* **95,** 351–358.
15. Engineer, F. N., and Sridhar, R. (1989) *Biochem. Pharmacol.* **38,** 1279–1285.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R, J. (1964) *J. Biol. Chem.* **193,** 265–275.
17. Buge, J. A., and Aust, S. D. (1979) *Methods Enzymol.* **52,** 302–310.
18. Halliwell, B., Gutteridge, J. M. C., and Aruoma, O. I. (1987) *Anal. Biochem.* **165,** 215–219.
19. MacColl, R., Williams, O., Eisele, L. E., and Berns, D. S. (1994) *Biochem. Biophys. Acta.*
20. Bairoch, A., and Apweiler, R. (2000) *Nucleic Acids Res.* **28,** 45–48.
21. Recknagel, R. O., and Ghoshal, A. K. (1967) *Pharmacol. Rev.* **19,** 145–208.
22. Recknagel, R. O., and Ghoshal, A. K. (1966) *Exp. Mol. Pathol.* **5,** 108–117.
23. Slater, T. F. (1966) *Nature* **209,** 36–40.
24. Aust, S. D., Chignell, C. F., Bray T. M., Kalyanaraman, B., and Mason, R. P. (1993) *Toxicol. Appl. Pharmacol.* **120,** 168–178.
25. Terry, M. J., Maines, M. D., and Lagarias, J. C. (1993) *J. Biol. Chem.* **268,** 26099–16106.
26. Stocker, R., Glazer, A. N., and Ames, B. N. (1987) *Proc. Natl. Acd. Sci.* **84,** 5918–1922.