C-Phycocyanin: A Biliprotein with Antioxidant, Anti-Inflammatory and Neuroprotective Effects

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Abstract: Phycocyanin (Pc) is a phycobiliprotein that has been recently reported to exhibit a variety of pharmacological properties. In this regard, antioxidant, anti-inflammatory, neuroprotective and hepatoprotective effects have been experimentally attributed to Pc. When it was evaluated as an antioxidant *in vitro,* it was able to scavenge alkoxyl, hydroxyl and peroxyl radicals and to react with peroxinitrite ($ONOO^-$) and hypochlorous acid (HOCl). Pc also inhibits microsomal lipid peroxidation induced by Fe^{+2}-ascorbic acid or the free radical initiator 2,2' azobis (2-amidinopropane) hydrochloride (AAPH). Furthermore, it reduces carbon tetrachloride (CCl_4)-induced lipid peroxidation *in vivo.* Pc has been evaluated in twelve experimental models of inflammation and exerted anti-inflammatory effects in a dose-dependent fashion in all of these. Thus, Pc reduced edema, histamine (Hi) release, myeloperoxidase (MPO) activity and the levels of prostaglandin (PGE_2) and leukotriene (LTB_4) in the inflamed tissues. These anti-inflammatory effects of Pc can be due to its scavenging properties toward oxygen reactive species (ROS) and its inhibitory effects on cyclooxygenase 2 (COX-2) activity and on Hi release from mast cells. Pc also reduced the levels of tumor necrosis factor (TNF-α) in the blood serum of mice treated with endotoxin and it showed neuroprotective effects in rat cerebellar granule cell cultures and in kainate-induced brain injury in rats.

Keywords: Phycocyanin, phycobiliprotein, Spirulina, antioxidant, free radical scavenger, anti-inflammatory, neuroprotective, hepatoprotective.

INTRODUCTION

Pc is one of the major pigment constituents of Spirulina, a microalgae used in many countries as dietary supplement whose nutritional and therapeutic values have been very well documented [1-3]. At present there is a mass of evidence in favor of the antioxidant properties of Pc which have been used to explain, al least in part, the anti-inflammatory effects also attributed to the protein.

It is well known that reactive oxygen species (ROS) are involved in a diversity of important pathological processes in medicine including among others: inflammatory and neurodegenerative diseases, atherosclerosis, cancer and reperfusion injury [4].

This review summarizes recent findings about the antioxidant, anti-inflammatory and neuroprotective effects of Pc and considers the potential benefits of Pc regular intake in the prevention of many pathological disorders associated with oxidative stress and inflammation.

SOME PHYSICAL-CHEMICAL PROPERTIES OF PC

Phycobiliproteins are a small group of highly conserved chromoproteins that constitute the phycobilisome, a macromolecular protein complex whose main function is to serve as a light harvesting complex for the photosynthetic apparatus of cyanobacteria and eukaryotic groups [5,6]. The most common classes of phycobiliproteins are allophycocyanin, phycocyanin and phycoerythrin all of which are formed by α and β protein subunits and carry different isomeric linear tetrapyrrole prosthetic groups (bilin chromophore) which differ in the arrangement of their double bonds. The bilin groups are attached to the polypeptides through thioether linkages to specific cysteinyl residues [7,8].

Pc is composed of two dissimilar α and β protein subunits of 17 000 and 19 500 Da, respectively, with one bilin chromophore attached to the α subunit (α 84) and two to the β subunit (β 84, β 155) [9].

Pc exists as a complex interacting mixture of trimer, hexamer and decamer aggregates. The relative amount of each species has been reported to be a function of pH, ionic strength, temperature and protein concentration [10]. The absorption spectrum of Pc monomer and all aggregates exhibit a strong first excited state band at 615 nm and a much weaker second excited state band at 360 nm. Both of these bands are due to the properties of the bilin chromophore. When the secondary, tertiary and quaternary structures of the protein are denatured, the visible absorption band as well as the fluorescence will drop in intensity [5].

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Pc is obtained from the microalgae cellular biomass by a freeze thawing process or by using a French pressure cell [11], and is purified by successive steps of ammonium sulphate precipitation and further DEAE-cellulose chromatography [12]. Pc is considered pure when the absorption ratio of visible maximum to 280 was greater than 4. Another criterion of purity was that allophycocyanin was not observed at 650 nm [13].

The chemical structure of the bilin chromophores in Pc (Fig. **1**) is very similar to bilirubin, a heme degradative product. Bilirubin is considered to be a physiologically important antioxidant against reactive species [14]. It inhibits oxidative modification of plasma proteins and aromatic amino acid residues. Scavenging of oxygen radicals by bilirubin has been shown to protect serum albumin as well as other biological targets [15].

ANTIOXIDANT PROPERTIES OF C-PHYCOCYANIN

The antioxidant properties of Pc, which have been mainly demonstrated by *in vitro* studies, are summarized in (Table **1**).

These studies have shown, by different experimental methods, that Pc is an efficient scavenger of oxygen free radicals and also reacts with other oxidants of pathological relevance such as HOCl and $ONOO^-$.

Many diseases are accompanied or even caused by oxidative stress that is characterized by excessive formation of ROS that cannot be counteracted by the antioxidant defense systems of the organism. Therefore the therapeutic use of natural or synthetic antioxidants appears to be promising.

Alkoxyl and Hydroxyl radical-Scavenging Activity of Pc

Scavenging of alkoxyl and hydroxyl radicals by Pc was demonstrated using a chemiluminescence (CL) assay [16].

Determination of alkoxyl radical scavenging activity of Pc was performed by measuring the inhibition of the CL produced by the reaction of *tert*-butyl hydroperoxide with ferrous ions in the presence of luminol. Trolox, a water-soluble analogue of vitamin E, was used as specific scavenger of these radicals. The results showed that Pc inhibits the CL in this system in a dose-dependent manner. A comparison with trolox indicated that 0.1 μM of trolox causes approximately the same effect as 2 μM of Pc in terms of 50 % inhibition of the CL produced.

The inhibition of CL produced by the Fenton reaction with luminol was used to evaluate the Pc scavenging capacity against hydroxyl radicals. In this system the CL signal was inhibited in a dose-dependent fashion by increasing Pc concentrations. It was reported that 24.7 μM of Pc caused the same inhibition (50%) as 1.6 mM of dimethyl sulfoxide a specific hydroxyl radical scavenger used as control.

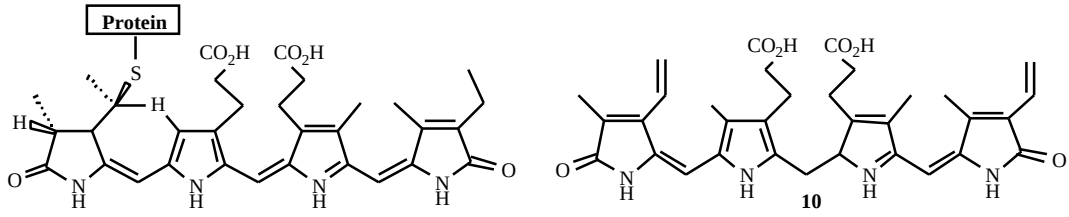
Double quenching experiments were done in each CL system in order to be sure that the effect of Pc was due to the scavenging of the desired oxygen free radical and not to the trapping of other free radical species.

Hydroxyl radical scavenging capacity of Pc has been also assayed by the inhibition of damage to 2-deoxyribose [16,17]. In this system Pc inhibited deoxyribose damage in a concentration-dependent fashion. The IC_{50} values reported for Pc using this method were 19 μM [16] and 28 μM [17]. The phycobiliprotein interacts with hydroxyl radicals with a reaction rate constant in the range of 1.9 to 3.5 x 10^{11} M^{-1} S^{-1}, whereas that obtained by the same method for some non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin and ibuprofen was (1.8 x 10^{10} M^{-1} S^{-1}) [18].

Bhat et al. [17] established the involvement of the bilin chromophore in the hydroxyl radical scavenging activity of the protein, studying its bleaching when it was incubated with a hydroxyl radical generating system (Fenton reagent) containing EDTA. Under these conditions, there was a significant decrease in the absorption at 618 nm (36.5% decrease). However when incubation was carried out in the absence of EDTA, a less significant (20%) decrease was also observed indicating a possible iron binding capacity of Pc. This iron binding capacity was subsequently confirmed by a fluorescence quenching experiment in which Pc interacted with iron ion with an association constant of 1.11 ± 0.06 x 10^5 M^{-1} [17].

Peroxyl Radical-Scavenging Activity of Phycocyanin.

Exposure of Pc to peroxyl radicals generated by thermolysis of AAPH leads to a progressive loss of the visible absorbance [19]. From an evaluation of the bilin groups destroyed per radical that interacts with the protein, it was concluded that the bilin moiety is the main target of the



a **b**

Fig. (1). Chemical structure of phycocyanin bilin chromophore (open-chain tetrapyrrol) (**a)** and bilirubin (**b**).

Table 1. Antioxidative Properties of Phycocyanin Demonstrated by *In Vitro* Studies

Reaction system	Effect	Author	Year	Ref.
Superoxide generated from Hypoxanthine-xantine oxidase	no effect	Romay *et al.*	1998	16
Alkoxyl radical generated from t-BOOH-ferrous sulphate	scavenge	Romay *et al.*	1998	16
Hydroxyl radical generated from Hydrogen peroxide-ferrous sulphate (Fenton Reagent)	scavenge scavenge	Romay *et al.* Bhat *et al.*	1998 2000	16 17
Peroxyl radical generated from AAPH thermolysis	scavenge scavenge	Lissi *et al.* Bhat *et al.*	2000 2000	19 17
Singlet oxygen	quench	Tapia *et al.*	1999	27
Lipid peroxidation induced by Fe^{+2}-ascorbic acid AAPH thermolysis	Inhibit inhibit	Romay *et al.* Bhat *et al.*	1998 2000	16 17
Peroxinitrite generated from Nitrite-acidified hydrogen peroxide	scavenge	Bhat *et al.*	2001	24
Hypochlorite	removed	Romay *et al.*	2000	26
Reactive oxygen production from neutrophils stimulated with opsonized zymosan	inhibit	Romay *et al.*	1998	16

radicals. Kinetic expressions were derived that allows an estimation of the substrate reactivity from the analysis of the rate of the bilin group modification as a function of the protein concentration. From this analysis it was concluded that micromolar concentrations of Pc are able to reduce the steady state concentration of the peroxyl radicals by one half, indicating a high antioxidant activity for this compound. This conclusion was confirmed by measuring the capacity of the protein to protect 1-naphthol from modification by peroxyl radicals. The results obtained showed that the bilin groups have, on a molar basis, an antioxidant activity similar to that of potent antioxidants such as catechin [19].

Bhat and Madyastha [17] also demonstrated the involvement of the bilin chromophore in the radical scavenging activity of Pc by studying the reactivity of the protein with peroxyl radicals derived from AAPH thermolysis. In this study, a significant decrease in the absorption at 618 nm (60 % decrease when 10 μM Pc was incubated with 10 mM AAPH at 37 °C) and a shift in the absorption maxima at 618 nm (21.5 nm shift) toward lower wavelength were noticed.

It was also shown that both native Pc and the reducing form (using $NaBH_4$) are able to scavenge peroxyl radicals. This was supported by the fact that when reduced Pc was incubated with AAPH (10 mM) at 37 °C there was a rapid decrease in the absorption at 418 nm with a concomitant appearance of peaks at 618 nm and 360 nm in the UV-visible spectrum indicating the oxidation of phycocyanorubin to phycocyanobilin by peroxyl radical [17].

These authors, using the competition kinetics of crocin bleaching by peroxyl radicals, also analyzed the interaction of peroxyl radical with Pc and its ability to scavenge this radical. These studies demonstrated that Pc 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 Pc and uric acid were of 1.54 and 3.5 respectively.

Phycocyanin bleaching by peroxyl radicals derived from thermal decomposition of AAPH and its protection by antioxidants has been proposed as a methodology to evaluate the oxygen radical absorbing capacity (ORAC) of pure compounds and/or complex mixtures [20].

Pc Effect on Lipid Peroxidation.

Lipid peroxidation mediated by ROS is believed to be an important cause of destruction and damage to cell membranes, because a simple initiating event can result in the conversion of hundreds of fatty acids side chain into lipid peroxides, which alters the structural integrity and biochemical functions of membranes [21]. It has been shown that Pc significantly inhibits the increase in lipid peroxides of rat liver microsomes after treatment with Fe^{+2}-ascorbic acid [16] or the free radical initiator AAPH [17].

Addition of Pc (200-540 μM) to isolated microsomes in the presence of Fe^{+2}-ascorbate resulted in a concentration-dependent decrease in thiobarbituric acid reactive substances (TBARS) as an index of hepatic lipid peroxidation [16]. The

calculated IC_{50} was 327 μM. Thus, Pc reduced both the rate and the final extent of lipid peroxidation.

This type of lipid peroxidation is caused by a Fenton-type reaction of the metal ion with traces of hydroperoxides, forming an alkoxyl radical and the higher valency state of the metal; the alkoxyl radical in turn propagates a free-radical chain reaction. Phycocyanin may inhibit this process at its earliest stage by removing the alkoxyl radicals or by binding the metal ion.

The Pc effect on peroxyl radical-induced lipid peroxidation in rat liver microsomes also has been studied [17]. It was demonstrated that Pc inhibits the azo-initiated microsomal lipid peroxidation in a concentration-dependent fashion with an IC_{50} value of 11.35 μM. Phycocyanin at 200 μM concentration inhibited nearly 95 % of peroxyl radical-induced lipid peroxidation. Reduced Pc also efficiently inhibited this reaction with an IC_{50} value of 12.7 μM. In fact both native and reduced Pc inhibited lipid peroxidation almost to the same extent.

In correspondence with these results it was demonstrated [17] that Pc also reduced CCl_4-induced lipid peroxidation *in vivo*. Intraperitoneal administration of Pc (50-200 mg/kg body wt) 3 h prior to CCl_4 treatment resulted in significantly lower production of malondialdehyde than was found in rats receiving only CCl_4. It is known that in CCl_4 intoxication, free radicals arising from its biotransformation induce lipid peroxidation. The trichloromethyl radical ($CCl_3^{\cdot}$) initially formed is relatively unreactive and this carbon-centered radical readily reacts with O_2 to form a peroxyl radical that is a good initiator of lipid peroxidation. Since it was demonstrated that Pc did not alter the liver function and the cytochrome P_{450} system [22], the protection by Pc against CCl_4-induced lipid peroxidation may not be related to a reduced formation of reactive metabolites of CCl_4, but due to the ability of Pc to scavenge peroxyl radicals.

It also has been reported [23] that Pc is able to protect human erythrocytes against lysis induced by peroxyl radicals. In this assay Pc (12-75 μM) inhibited erythrocyte haemolysis in the same way as trolox and ascorbic acid, well-known antioxidants. Based on IC_{50} values Pc proved to be almost sixteen times more efficient as an antioxidant than trolox and about twenty times more efficient than ascorbic acid.

Peroxynitrite Scavenging Activity of Phycocyanin.

The interaction of Pc and its bilin chromophore with peroxynitrite ($ONOO^-$) was studied spectroscopically by Bhat and Madyastha [24]. They demonstrated that the addition of increasing concentrations of $ONOO^-$ (0-200 μM) to Pc (10 μM) significantly decreased its absorption at 618 nm, with no change in the absorption at 360 nm. Nearly 50 % of absorption at 618 nm was lost in the presence of 200 μM $ONOO^-$, although there was no shift in the absorption maxima.

The spectra of bilin chromophores are characterized by absorption maxima at 610 and 365.5 nm. The addition of $ONOO^-$ (0-125 μM) to bilin chromophores (10 μM) decreased the absorbance peak at 610 nm and 365.5 nm, with a shift towards lowers wavelength (563 and 329.5 nm) at lower concentrations of $ONOO^-$. At higher concentration of $ONOO^-$ (125 μM) there was no further shift in the absorption maxima and the chromophore was almost completely bleached.

The scavenging of $ONOO^-$ by Pc and its bilin chromophore was also evaluated using competitive kinetics of pyrogallol red bleaching assays [24]. Pyrogallol red is one of the more efficient dyes that can be used to evaluate the $ONOO^-$ scavenging activity of any compound in aqueous solution.

Pc is an efficient scavenger of $ONOO^-$; at 70 μM concentration it inhibited pyrogallol red bleaching to the extent of nearly 90 %. However, both bilin chromophore and glutathione (a known $ONOO^-$ scavenger) appeared to be more efficient scavengers of $ONOO^-$ at lower concentrations than Pc. It was also noticed that Pc, its bilin chromophore and glutathione, inhibited bleaching of pyrogallol red in a concentration-dependent manner with an IC_{50} value of 21.8 ± 2.6 μM, 30.5 ± 0.8 μM and 4.8 ± 1.2 μM respectively. The relative antioxidant activity ratios (k_a/k_{PR}) calculated for Pc, its bilin group and glutathione were 3.9, 1.8 and 5.2 respectively. The relative antioxidant ratio as well as IC_{50} value clearly suggested that Pc is more efficient $ONOO^-$ scavenger than its bilin chromophore. This result was attributed to the interaction of $ONOO^-$ with tyrosine and tryptophan residues present in the apoprotein moiety.

The authors also proved that the bilin chromophore significantly inhibits the $ONOO^-$-mediated single-strand breaks in supercoiled plasmid DNA in a dose-dependent manner with an IC_{50} value of 2.9 ± 0.6 μM. Several anticarcinogenic agents are known to inhibit oxidative DNA damage and tumor promotion [25] and so it is quite possible that Pc may act as an anticarcinogenic agent.

Pc Reaction with Hypochlorite.

Kinetics and mechanism of Pc reaction with HOCl were established by measuring the decrease in the visible band absorbance (620 nm) at different HOCl/protein ratios [26]. It was concluded that HOCl bleaches Pc visible absorbance with a second-order rate constant (pH 7.4) of 1.3 x 10^3 M^{-1} s^{-1}. Over all the conditions employed it was observed that in excess of Pc, 0.16 ± 0.04 chromogenic groups were bleached per HOCl molecule introduced into the system, which implies that apoprotein moiety, and not the chromogenic groups, is the main target of the HOCl attack. In this regard, the behavior of Pc is similar to that reported for its reaction with singlet oxygen [27] and contrast with results obtained employing peroxyl radicals [19] or 2,2' azinobis (3-ethylbenzthiazoline sulfonic acid (ABTS) derived radicals (C. Aliaga unpublished results).

The reaction of apoprotein with HOCl was mainly related to the presence of about nine methionine groups in this molecule, which can efficiently react with HOCl.

From the rate of bilin group modification and the fraction of reaction at these groups, it was concluded that the total rate of Pc reaction with HOCl takes place with a rate constant k_{Pc} of 2.5 x 10^4 M^{-1} s^{-1} when the rate is expressed in concentration of Pc monomeric units. Although HOCl and/or its deprotonated anion (OCl^-) play an important role in the immune defense system against microorganisms and also in inflammation, the rate of Pc reaction with HOCl is too slow to provide protection to other biomolecules under physiological conditions.

Effects of Phycocyanin on Neutrophil Activation.

CL of polymorphonuclear leukocytes is the final result of luminol oxidation by strong oxidants, such as oxygen radicals and peroxides, emanating from enzymatic reactions. For addition to the MPO-H_2O_2 halide system, the release of arachidonic acid (AA) by phospholipase A_2 and of diacylglycerol and inositol trisphosphate by phospholipase C, the metabolism of AA by the cyclooxygenase (COX) and lipoxygenase (LOX) pathways, the activation of membrane NADPH oxidase by diacylglycerol and calcium mobilization by inositol trisphosphate are all able to induce the CL reaction. Inhibition of any of these mechanisms suppresses the CL response [28].

It was recently demonstrated [16] that Pc inhibited luminol-dependent CL of neutrophils stimulated with opsonized zymosan. In this model system Pc (27-81 μM) reduced significantly the CL signal, expressed as the mean of the area under the curve. This behavior was explained through the capacity of Pc to scavenge free radicals and peroxides arising during the respiratory burst of phagocytic cells. However other mechanisms by which Pc can inhibit CL in this system, have been not yet clarified.

ANTI-INFLAMMATORY EFFECTS OF PHYCOCYANIN

Pc was first tested in an experimental model of inflammation in which the inflammatory response was induced by peroxide in the mouse paw [16]. It is a suitable *in vivo* model to test agents that induce scavenging effects against H_2O_2 and $OH^{\cdot}$ radicals. Glucose oxidase injected into the mouse paw reacts with endogenous glucose and generates H_2O_2, which subsequently produces $OH^{\cdot}$ radicals; both together are responsible for tissue damage and for the accompanying inflammatory changes [29,30].

Pc at doses of 100 and 200 mg/kg p.o significantly inhibited paw edema in a dose-dependent fashion in this test. This anti-inflammatory effect can be due, at least in part, to the scavenging of hydroxyl radicals, taking into account that dimethyl sulfoxide, a well known scavenger of $OH^{\cdot}$, also inhibited the inflammatory response [16]. Therefore, the scavenging action of Pc against $OH^{\cdot}$ seems to be relevant to its anti-inflammatory effects.

Taking into account the former findings, it was decided to research the potential activity of Pc in some experimental models in which both ROS and AA metabolites are involved in the induction and development of the inflammatory response. The oral administration of Pc at doses of 50-300 mg/kg led to anti-inflammatory activity in the models tested, although its effect varied (Table **2**).

It was effective in the carrageenan-induced rat paw edema (Table **2**), which is a suitable model for the *in vivo* assessment of COX inhibitors [31] and also of novel antioxidants [32]. These authors have provided evidence that oxygen radicals play an important role in the maintenance of carrageenan paw edema. On the other hand, it was demonstrated that the anti-inflammatory activity of Pc is not

Table 2. Anti-inflammatory and Protective Effects of Pc in Laboratory Animals

Experimental model	Species	Dosage (mg/kg)	Reference
Glucose oxidase-induced inflammation in paw	Mouse	200 p.o	Romay *et al.* 1998 (16)
Carrageenan-induced paw edema	Rat	200 p.o	Romay *et al.* 1998 (33)
Arachidonic acid-induced ear edema	Mouse	50 p.o	Romay *et al.* 1998 (33)
CCl_4 induced liver damage	Rat	200 i.p	Vadiraja *et al.* 1998 (22)
Endotoxin-treated animals	Mouse	200 p.o	Romay *et al.* 2001(47)
Zymosan-induced arthritis	Mouse	25 p.o	Remirez *et al.* 1999 (58)
Acetic acid-induced colitis	Rat	150 p.o	González *et al.* 1999 (49)
Ovalbumin-induced ear edema in sensitized animals	Mouse	200 p.o	Remirez *et al.* 2002 (68)
Skin reactions to histamine and compound 48/80	Rat	200 p.o	Remirez *et al.* 2002 (68)
Kainate-induced brain injury	Rat	100 p.o	Rimbau *et al.* 1999 (70)
Tetradecanoyl phorbol acetate-induced ear edema	Mouse	300 p.o	Romay *et al.* 1998 (33)
Cotton pellet granuloma	Rat	200 p.o	Romay *et al.* 1998 (33)

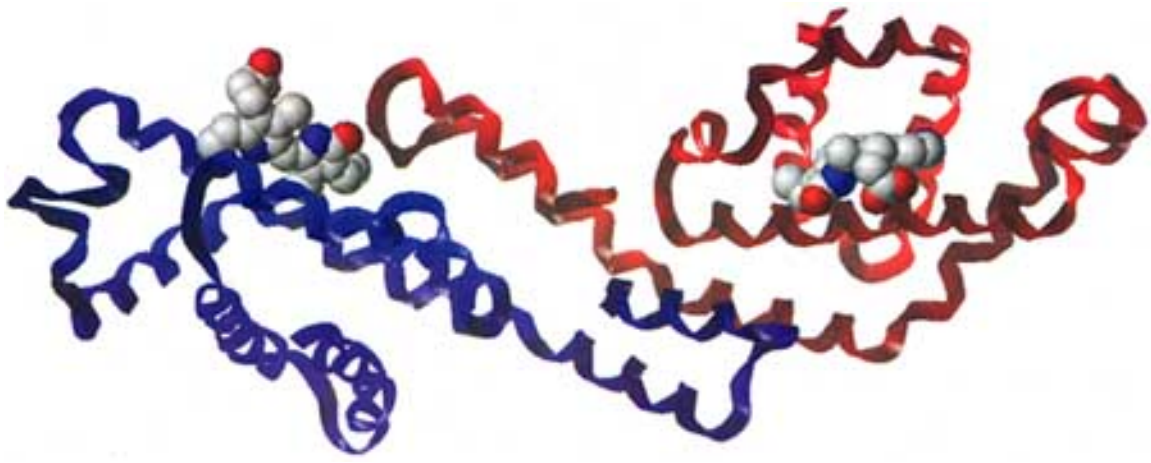


Fig. (2). Structure of the α,β- dimer of phycocyanin from *Spirulina platensis* at 2.2 Å resolution [K. Brejc, R. Ficner, R. Huber, S. Steinbacher, J. Mol. Biol., 249, 424 (1995). [Figure prepared by Editor-in-Chief].

dependent on corticosteroid release, because it inhibited carrageenan-induced rat paw edema to the same extent in intact and adrenalectomized rats, when otherwise identical treatment regimens were used [33].

The anti-inflammatory activity of Pc was greater in the AA-induced mouse ear edema (Table **2**) [33] which is considered to be a suitable test for the detection of COX and/or LOX inhibitors of AA metabolism [34].

Crummey *et al* [35] also demonstrated that antioxidants such as butylated hydroxyanisole, α-tocopherol, retinoic acid and desferrioxamine are able to strongly inhibit this test. They postulated that the effect might be due to a direct reduction of both enzymatic and non-enzymatic lipid peroxidation (and hence AA metabolism) as well as to a further reduction in the activity of COX and LOX due to their requirement for (hydro)-peroxides to stimulate enzymatic function.

More recently, Maccarrone et al [36] provided further evidence in favor of this mechanism of action for the chain-breaking antioxidants, ascorbic acid and trolox, which inhibited soybean LOX in the micromolar concentration range. It is not surprising since lipoxygenation occurs via a carbon centered radical, thus compounds that inhibit the formation of this radical or trap it once formed would be expected to be LO inhibitors.

In accordance with this view, it was found that Pc (50-200 mg/kg p.o) inhibited in a dose-dependent manner edema as well as PGE_2 and LTB_4 levels in the mouse ear treated with AA [37,38] most probably by a mechanism mentioned above.

The greater anti-inflammatory effect of Pc in AA-induced mouse ear inflammation, with respect to that of carrageenan-induced edema, seems to be related to the dual inhibitory action of Pc on both pathways of AA metabolism (prostaglandins and leukotrienes) [37,38], which is relevant for the former model whereas in the carrageenan test the role of leukotrienes in the inflammatory response is minor [32].

In line with these results Reddy et al [39], using two related assays systems (isolated enzyme assay and whole blood assay), demonstrated that Pc is a selective inhibitor of COX-2. In the isolated enzyme assay using human recombinant COX-2 the IC_{50} value obtained for the inhibition of COX-2 by Pc was much lower (180 nM) as compared to those of the drugs celecoxib (255 nM) and rofecoxib (401 nM), two well-known selective COX-2 inhibitors.

In the human whole blood stimulated by lipopolysaccharide (LPS) and measuring the release of PGE_2, Pc very efficiently inhibited COX-2 with an IC_{50} value of 80 nM even lower than the value obtained with the partially purified enzyme. Furthermore, Reddy et al [39] provided strong evidence that the apoprotein but not the bilin chromophore, plays a key role in the selective inhibition of COX-2. They argued that the active site of COX-2 is larger than that of COX-1 so that it can accommodate bigger structures. Phycocyanin is significantly larger in size (37.5 KDa) than NSAIDs. Its three-dimensional structure probably would facilitate the proper binding with the active site of COX-2. In agreement with this view, the chromophore of Pc lost COX-2 selectivity, which supports the proposed hypothesis.

The inhibition of COX-2 by Pc also seems to be involved in its hepatoprotective effect on CCl_4-induced liver damage [22]. One of the main processes involved in this model is the free radical-catalyzed lipid peroxidation [41], which is accompanied by activation of COX-2 and increased synthesis of prostaglandins [42]. Thus the hepatoprotective effect of Pc could be due to its ability to efficiently scavenge free radicals and inhibit lipid peroxidation [16,17] as well as COX-2 activity [39].

Very recently, Remirez et al [42] have provided strong evidence supporting an important role of Kupffer cells (KC), in the hepatoprotective effect of Pc. These authors performed experiments in the isolated perfused mouse liver and it was demonstrated that Pc (0.25 mg/ml) decreases the respiratory activity of the liver induced by colloidal carbon infusion. This response mainly represents the respiratory burst activity of KC involving NADPH oxidase and NOS activities [43], with secondary participation of mitochondrial respiration of KC for energy supply for carbon phagocytosis [44] and the stimulation of O_2 uptake at the parenchimal cell level, possibly mediated by prostaglandins released by activated liver macrophages [45]. Furthermore, diminution of carbon-induced respiratory burst activity by Pc is accompanied by a significant decrease in the carbon-induced sinusoidal release of lactate dehydrogenase, which reflects the integrity of the plasma membrane of hepatocytes and of other cells residing in the liver and therefore the hepatoprotective effect [46].

Recently, the Pc effects on TNF-α and nitrite levels in serum of mice treated with LPS were examined [47]. TNF-α, measured by cytotoxicity on L-929 cells 1h after LPS injection (0.5 mg/kg i.p), was significantly and dose-dependently reduced in serum by Pc (50-300 mg/kg p.o) treatment. Nitrite was measured in serum by the Griess reaction 18 h after LPS (30 mg/kg i.p); it was also reduced dose-dependently by Pc pretreatment (100-300 mg/kg p.o).

The inhibitory effects of antioxidant agents in this experimental model have been ascribed by various authors to inhibition of activation of nuclear transcription factor ($NF_{\kappa B}$) which is activated by ROS with the subsequent induction and expression of various cytokines and enzymes such as TNF-α and iNOS, respectively, which are involved in the induction and development of endotoxic shock [48]. In agreement with these results, Remirez et al [42] recently found in rats subjected to thyroid hormone (T_3) administration that Pc reduced TNF-α and nitrite levels in serum and liver iNOS activity. Therefore, in both experimental models inhibition of activation of $NF_{\kappa B}$ seems to be involved in Pc mode of action.

Pc was also pharmacologically evaluated in acetic acid-induced colitis in rats [49], an experimental model of inflammatory bowel disease (IBD) in which colitis is induced by enemas of 1 mL of 4 % acetic acid. In this experimental model Pc at doses of 150, 200 and 300 mg/kg p.o significantly inhibited MPO activity, which indicates a reduction of neutrophil infiltration in colonic tissue. In agreement with these results the histological and ultrastructural findings also demonstrated protective and anti-inflammatory effects of Pc in rat colon. 5-aminosalicylic acid (5-ASA) at a dose of 200 mg/kg, used as reference drug, exerted anti-inflammatory effects comparable with those of Pc [49].

Grisham [50] and others [51,52] have provide strong evidence that ROS such as superoxide, hydrogen peroxide, hydroxyl radical and HOCl are involved as mediators of experimental and human IBD. Also, nitric oxide (NO) and $ONOO^-$ have been suggested as potential mediators of colitis induction [53,54]. Taking into account the antioxidative effects of Pc aforementioned, it is conceivable that its anti-inflammatory activity in colitis can be due, at least in part, by scavenging of ROS. The beneficial effect of 5-ASA in IBD has been also ascribed to its potent antioxidative effect [55]. Other potential mediators that can be implicated in the anti-colitis effect of Pc are TNF-α, other cytokines and leukotrienes, especially LTB_4, which are increased in the inflamed colonic mucosa of IBD [56] and experimental colitis [57]. LTB_4 has potent chemotactic properties on neutrophils and Pc exerts inhibitory effects on the release of this mediator [38].

Pc was also evaluated in zymosan-induced arthritis in mice [58], an experimental model of rheumatoid arthritis in which complement is activated via alternative pathway and the secretion of lysosomal enzymes into the knee joint synovial fluid is induced. This activity correlates with histomorphological changes observed in the joint, such as vasculitis, synovitis and pannus formation.

It is increasingly recognized that ROS such as hydroxyl radicals, hydrogen peroxide and HOCl are involved in rheumatoid arthritis [59]. In accordance with it, some antioxidant compounds [60-62] have provided protective effects in some arthritis models and has been ascribed to its antioxidant properties. Zymosan is well known as a powerful releaser of AA metabolites that contribute actively to the early phase of inflammatory reaction [63]. Furthermore, TNF-α and other cytokines are well known mediators of zymosan-induced arthritis and have been found in the synovial fluid [64,65]. In this context and using the experimental conditions described elsewhere [58], Pc (25, 50 and 100 mg/kg, p.o) administered on a daily dose for 8 days, suppressed the activity of the lysosomal enzyme β-glucuronidase by 60, 80 and 93 % with respect to the mice treated with zymosan alone [58]. Furthermore, the histopathological and ultrastructural studies performed in the knee joints of mice treated with Pc or the reference drug, triamcinolone, showed a decrease in the inflammatory response. There was no destruction of general joint architecture or pannus formation and the reduction of bone erosion was pronounced, whereas ultrastructural studies showed well-preserved chondrocytes in contrast with severe alterations in tibial articular cartilage in animals treated with zymosan alone [58]. Taking into account that Pc inhibits ROS and AA metabolism as well as TNF-α in mouse serum [16,37,38], it is demonstrable that these are the targets of the phycobiliprotein pharmacological activity as anti-inflammatory and anti-arthritic agent.

Very recently it was demonstrated that Spirulina under the same experimental conditions above mentioned and at doses four time greater than Pc (which correlates with 20-25 % that represents Pc in the composition of Spirulina) exerted both similar anti-inflammatory and anti-arthritic effects in zymosan-induced arthritis in mice [66]. These results provide evidence that Pc is the main anti-inflammatory and anti-arthritic agent of the microalgae.

An inhibitory effect of Spirulina [67] and Pc [68] on allergic inflammatory reactions has also been reported. The anti-allergic effect of Pc was demonstrated in experimental models of ovalbumin-induced ear swelling in sensitized mice, skin reactions to Hi and compound 48/80 in rats, and

Hi release by compound 48/80 from isolated rat peritoneal mast cells. In all of them Pc (100-300 mg/kg p.o) significantly inhibited in a dose dependent fashion the parameters evaluated such as edema, MPO activity, skin reactions and Hi release. Taken together these findings support the potential usefulness of Pc in allergic inflammatory diseases; for that reason it merits further pharmacological studies to confirm this view.

NEUROPROTECTIVE EFFECTS OF PHYCOCYANIN

Pc (1-3 mg/ml) prevents cell death caused by 24 h potassium and serum (K/S) withdrawal in rat cerebellar granule cell (CGC) cultures [69]. After 4 h K/S deprivation, Pc inhibited ROS formation measured as 2',7'-dichlorofluorescein fluorescence, showing its scavenger capability. Also pre-treatment of CGC cultures with Pc reduced thymidine incorporation into DNA below control values and reduced dramatically apoptotic bodies as visualized by propidium iodide, indicating inhibition of apoptosis induced by K/S deprivation.

Flow cytometry studies indicated that 24 h K/S deprivation acts as a proliferative signal for CGC, which show an increase in S-phase percentage, and cells progressed into the apoptotic pathway. Pc protected CGC from apoptosis induced by K/S deprivation.

The neuroprotective role of Pc was also examined *in vivo* [70] on the neurobehavioral and neuronal damage induced in rats by kainic acid (KA). Classic effects of KA administration, including "wet dog shakes", tremors and seizures, were taken as signs of alterations in neurobehavior. In addition, changes in body weight were also measured in the different groups.

The incidence of neurobehavioral changes was significantly lower in rats receiving Pc (100 mg/kg p.o). These animals also gained significantly more weight than those ones only receiving KA, whereas their weight gain did not differ significantly from controls. Equivalent results were found when the neuronal damage in the hippocampus was evaluated through changes in peripheral benzodiazepine receptors (microglial marker) and heat shock protein 27kD expression (astroglial marker).

At present, the mechanisms by which Pc exerts its neuroprotective effects are not clear. However, our results and growing evidence support the hypothesis that the phycobiliprotein, acting as an antioxidant, inhibits neuronal death by a mechanism that involves free radicals scavenging and therefore Pc may be useful for the treatment of some neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases [71].

PHYCOCYANIN TOXICITY

Although the effective dosage range of Pc in various animal models of inflammation was from 25 to 300 mg/kg p.o, the safety of the phycobiliprotein seems to be high. Thus unlike other NSAIDs, Pc seems to possess low toxicity in rats and mice and lack of adverse effects [33]. The measured LD_{50} values were estimated to be greater than 3 g/kg for rats and mice. No mortality was induced even at the highest dose of Pc tested (3 g/kg p.o). The animals were observed for 14 days and neither alteration in behavior nor statistical differences in body weight were found between treated and non-treated animals. In the histopathological studies no damage to organs or tissues was found. However, further preclinical pharmacological and toxicological studies are required to determine the safety of Pc as potential drug. Also pharmacokinetic studies and of metabolism must be performed to the phycobiliprotein as previous stages to clinical trials.

CONCLUDING REMARKS

Among the antioxidative agents Pc appears to be promising. It exerts also anti-inflammatory, neuroprotective and hepatoprotective effects, which are closely connected with its antioxidative activity.

The bilin chromophore seems to be the main target of Pc reaction since the *in vitro* radical assisted bleaching of chromophore in Pc, clearly indicates its involvement in the scavenging of ROS. However, the interaction of the apoprotein moiety with oxidant species like $ONOO^-$ and HOCl have been also demonstrated. Pc can react then at both levels, the prosthetic group and the apoprotein.

On the other hand, when Pc is orally administered it must suffer degradation in the gastrointestinal tract and metabolites of lower molecular weight than Pc must be involved in the observed pharmacological effects.

The favorable characteristics of Pc are mainly as follows: 1) a pronounced cyto- and tissue-protective potential, in particular against oxidative stress which is strongly involved in inflammatory, neurodegenerative, cancer and reperfusion injury disorders among others; 2) an anti-inflammatory potential which is due to a set of multi-site actions such as, scavenging of various ROS, anti-lipoperoxidative and inhibitory effects on both pathways of AA metabolism (COX-2 inhibitor), inhibition of Hi release from mast cells and inhibitor of TNF-α release; 3) low toxicity in rats and mice; 4) taking into account that Pc is a major constituent of microalgae Spirulina (20 % of algae dry weight), it might exert therapeutic effects when it is administered alone or included in the microalgae used as dietary supplement.

ABBRIVIATION

AAPH	=	2,2' azobis (2-amidinopropane) hydrochloride
AA	=	Arachidonic acid
CCl_4	=	Carbon tetrachloride
$CCl_3^{\cdot}$	=	Trichloromethyl radical
CGC	=	Cerebellar granule cells

CL = Chemiluminescence

COX-2 = Cyclooxygenase 2

DMSO = Dimethyl sulphoxide

INOS = Induced nitric oxide synthase

KC = Kupffer cells

KA = Kainic acid

LOX = Lipoxygenase

LPS = Lipopolysaccharide

LTB_4 = Leukotriene B_4

MPO = Myeloperoxidase

$NF_{\kappa B}$ = Nuclear transcription factor

NO = Nitric oxide

NSAIDs= Non-Steroidal anti-inflammatory drugs

ORAC = Oxygen radical absorbing capacity

$ONOO^-$ = Peroxinitrite

Pc = Phycocyanin

PGE_2 = Prostaglandin E_2

ROS = Reactive oxygen species

TBARS = Thiobarbituric acid reactive substances

TNF-α = Tumor necrosis factor-α

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