Protective effects of C-Phycocyanin against lipid peroxidation of serum lipoproteins and hepatic microsomes.

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Summary

Lipid peroxidation has been considered as deleterious phenomenon associated with diseases such as atherosclerosis and neurodegeneration. C-phycocyanin, the main active compound of the blue-green algae *Spirulina platensis*, has well known antioxidant properties, but its effect on lipid peroxidation has not been extensively studied. Therefore, hepatic microsomes peroxidation and lipoprotein oxidation resistance studies were undertaken and biomarkers of lipid peroxidation, such as malondialdehyde, 4-hydroxy-alkenals, total hydroperoxides, conjugated dienes and peroxidation potential were spectrophotometrically determined. Our results show that c-phycocyanin has a significant inhibitory effects on lipid peroxidation ($p<0.05$), demonstrated by the inhibition of 80.3 % of 4-hydroxy-alkenals formation, 75.3 % susceptibility to lipid peroxidation and 47.0 % total hydroperoxides generation. This natural product conferred a significant ($p<0.05$) protection of plasma lipoproteins to copper/ascorbate-induced oxidation and our data show that it acts as a powerful lipid-phase antioxidant, reinforcing the current knowledge on the use of c-phycocyanin as an alternative therapy for oxidative stress linked pathological conditions.
Key words: Lipid peroxidation, c-phycocyanin, antioxidant capacity, oxidative stress.

Introduction

Lipid peroxidation (LPO) was first studied in the 1930's in relation to food deterioration, but since then there has been increasing evidence showing the involvement of free radicals in biology, leading to renewed attention on LPO with a wider scope in the fields of chemistry, biochemistry, nutrition and medicine [1,2], among others. Later studies revealed that, like proteins, carbohydrates, and nucleic acids,

lipids are targets of reactive oxygen species (ROS) and became oxidized to render cytotoxic products [3]. Due to the role of LPO in the pathogenesis of cardiovascular, neurodegenerative and other chronic diseases, it is of major importance to evaluate natural products that can potentially inhibit or block this reaction.

C-phycocyanin (c-Pc) is a biliprotein of the blue-green alga *Spirulina platensis*. This protein contains a tetrapyrrole phycocyanobilin, which is covalently attached to the apoprotein and is considered as the major responsible of its important antioxidant properties [4]. It has been reported that c-Pc has significant antioxidant and radical-scavenging properties, offering protection against oxidative stress [5]; nevertheless the effects of c-Pc on LPO of lipoproteins and hepatic microsomes have not been extensively evaluated.

Hepatic microsome LPO [6,7] is a well known system for evaluating the antioxidant potential of a substance, a setting that could offer important information on the effects of the antioxidant activity of c-Pc, enabling the estimation of several LPO-derived products.

On the other hand, the characteristic absorbance of conjugated dienes (CD) has been used for continuous monitoring of LPO in lipoproteins [8]. The method provides continuous oxidation curves with a typical three-phasic shape from which several parameters may be drawn. One of these parameters is the maximal rate of propagation (maximal optical density in relation to the higher CD amount), which is associated to oxidation sensitivity of lipids in the absence or presence of antioxidants [9]. The length of LPO inhibition (lag-phase) is related to lipoprotein oxidation resistance. It is estimated as the time required to consume chain-breaking antioxidants present in the reaction mixture and can be used as a LPO inhibition marker [10]. Using these procedures we studied the c-Pc effect on oxidative lipid damage to show its protective effect on the hepatic microsomes and serum lipoproteins LPO.

Materials and methods

Reagents

All reagents were purchased from Sigma-Aldrich (St Louis, MO, USA).

Purification of C-phycocyanin

All procedures were carried out at 4 °C, in the dark. Fresh *Spirulina platensis* cells were resuspended in 20 mM Tris, pH 8.0 (0.1g of cells/mL). Cells were sonicated (70 % amplitude and cycle 1.0) two times for 1 min (IKA Werke GmbH&Co. KG., Germany). Proteins were precipitated with ammonium sulphate as previously described [11]. C-Pc was purified using aqueous-two phase extraction as reported by Patil *et al.* [12]. The upper phase was diluted 3-fold with 50 mM Tris pH 8.0 and loaded onto a Q-Sepharose Fast Flow column (Ø =1.6 cm; h = 5.0 cm, GE Healthcare, USA), previously equilibrated in the same buffer.

The column was washed with 50 mM Tris, 60 mM NaCl, pH 8.0 and then with 50 mM Tris, 120 mM NaCl, pH 8.0. C-Pc eluted in 50 mM Tris, 240 mM NaCl, pH 8.0 and was precipitated by adding ammonium sulfate at 50 % saturation. The pellet was resuspended in ammonium sulphate at 20 % saturation and applied onto a butyl column (Ø = 1.6 cm; h = 5.0 cm, Tosohaas, Japan), equilibrated in the same buffer. The column was washed with ammonium sulphate at (i) 15 % and (ii) 10 % saturation and c-Pc was eluted in 20 mM Tris pH 8.0.

Spectroscopic measurements

An absorbance spectrum performed in the range of 250 to 700 nm is shown in Figure 1A. The purity of c-Pc was determined by the absorbance ratio A_{620}/A_{280} [13] and the concentration of c-Pc was calculated according to Lambeer-Beer's law considering its reported extinction coefficient [14].

Polyacrylamide gel electrophoresis

SDS-PAGE in 15 % polyacrylamide gel was carried out as described previously [15] at 30 mA in a miniProtean chamber (BioRad, USA). Proteins were detected with Coomassie Brilliant Blue R-250 (Figure 1B).

Animals

Male Sprague-Dawley rats, weighing 250-300 g were obtained from CENPALAB (La Habana, Cuba) and then adapted to laboratory conditions (60% humidity, 25 ± 1 ºC) for at least 1 week before the experiments. The animals were housed in groups of five, maintained on a 12 h light/darkness cycle, with free access to food and water before and after the surgery. All procedures were performed in compliance with the Institutional Animal Care Committee and with the European Communities Council Directive of 24 November 1986 (86/609/EEC) for animal experimentation.

Rat liver microsomes preparation

The rats were sacrificed by decapitation after an overnight fast. The liver was removed and processed in a tissue homogenizer (Edmund Bühler LBMA, Germany), after which liver microsomes were isolated as described elsewhere [6] and stored at -80°C. Protein concentration in the microsomal suspension was determined according to Bradford [16].

Lipid peroxidation assay in rat liver microsomes

The microsomes suspension was preincubated with 0.4 mM ADP and 10 μM $FeSO_4$ at 37°C for 5 min before each compound to be tested was added. After a further 5 min incubation, concentrations of 10, 20 and 40 μM of c-Pc or Trolox C were added to the reaction mixture and then, LPO was initiated by adding 500 μM ascorbic acid [17]. After a proper time of incubation, the LPO was assayed determining malondialdehyde (MDA) concentration as previously described [18]. The extent of peroxidation was expressed as the change in absorbance of reaction mixture at 586 nm.

On the other hand, the microsomes suspension was incubated with a solution of 2 mM copper sulfate and 40 μM of c-Pc or Trolox-C at 37 ºC for 24 h. The peroxidation potential (PP) was calculated according Ozdemirler *et al*. [19]. The levels of 4-hydroxyalkenals (4-HA) were determined at 586 nm as described previously [20], while total hydroperoxides (TH) levels were measured by the Bioxytech H_2O_2-560 kit following manufacturer's instructions (Oxis International Inc., USA).

Oxidation resistance of plasma lipoproteins assay

Blood samples (3 mL) were obtained from abdominal aorta and mixed with 36 mM sodium citrate, after a gentle homogenization the samples were immediately centrifuged at 1000 *g*, at 4 ºC for 4 min. The supernatant was collected and 40 μM of c-Pc or Trolox-C were added; kinetics of CD formation was spectrophotometrically monitored at 245 nm as described previously [21]. All biochemical parameters were determined using a Pharmacia 1000 Spectrophotometer (Pharmacia LKB, Sweden).

Results

C-phycocyanin

C-Pc had an absolute absorbance maximum around 620 nm and a relative maximum around 350 nm (Figure 1A) as reported previously [12,22]. The purity of c-Pc, measured as the absorbance ratio at 620 nm and 280 nm, was 4.58. The SDS-PAGE showed only two bands corresponding to characteristic alpha and beta chains of c-Pc.

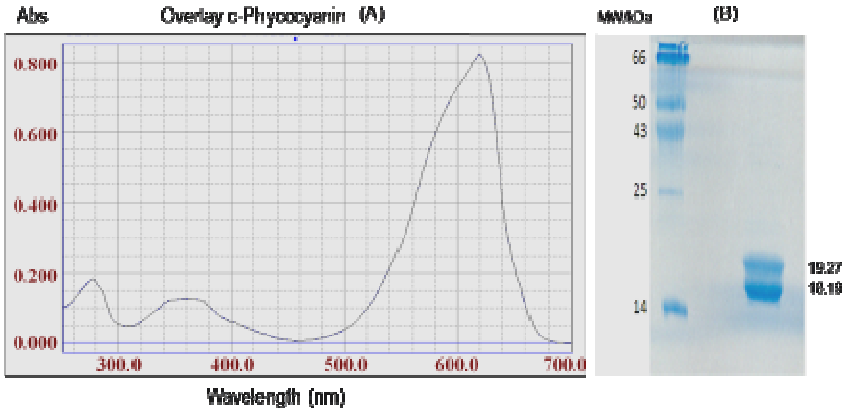

Figure 1. Evaluation of C-Pc purity obtained through (A) Absorption spectra and (B) SDS-PAGE using Comassie blue staining. (A): Absorption spectrum and (B) SDS-PAGE analysis. The protein preparation had a characteristic maximum of absorption at 620 nm and its ratio A_{620}/A_{280} was greater than 4.

Inhibition of LPO in hepatic microsomes

The LPO, induced in hepatic microsomes, was notably reduced by c-Pc as shown in Figure 2. A significant ($p<0.05$) inhibition of oxidative damage on lipids was noted: more than 50 % for 10 and 20 µM, and 80 % for 40 µM of c-Pc respect to non-inhibited reaction (control) and 42 % respect to Trolox-C-treated samples at the same concentration.

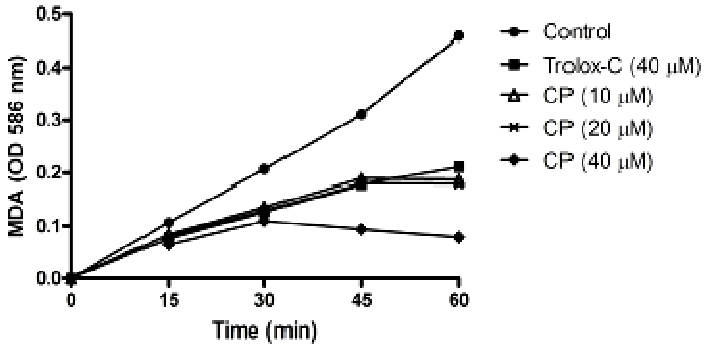

Figure 2. Inhibitory effects of c-Pc on LPO in hepatic microsomes. The inhibitory effect of c-Pc on microsome LPO was monitored every 15 minutes during 60 minutes. CP: samples treated with c-

phycocyanin, OD: optical density (corresponding to MDA formation). Each point represents the mean of three separate experiments which varied by no more than 10%.

Effects of c-Pc on LPO biomarkers

The treatment with c-Pc was able to reduce the LPO-derived substances (Table 1). Our data showed that 40 µM of c-Pc significantly inhibited ($p<0.05$) the formation of 4-HA and TH. The results demonstrated that c-Pc inhibited the formation of 4-HA in 80.33%, while Trolox-C did so in 79.72%. In control samples there was detected a high concentration of TH, while c-Pc reduced the levels of these oxidation products in a 47%, similar behaviour was observed in those samples in which 40 µM of Trolox-C were added.

LPO biomarkers	Control	Trolox-C (40 µM)	C-Pc (40 µM)
PP, µM of MDA/mgPr	9.41 ± 1.15	3.34 ± 0.95*	2.32 ± 0.86*
4-HA, µM/mgPr	4.98 ± 0.86	1.01 ± 0.38*	0.98 ± 0.19*
TH, µM/mgPr	34.25 ± 4.87	16.64 ± 1.83*	18.20 ± 2.12*

Table 1. Values represent the mean ± SD of five determinations in hepatic microsomes. Asterisks mean statistical differences ($p<0.05$) respect to control. Trolox-C was used as antioxidant standard. The concentration of different lipid peroxidation biomarkers is expressed per milligrams of total proteins (Pr).

Effects of c-Pc on plasma lipoprotein oxidation

Finally, a protective effect on lipids contained in lipoproteins of 40 µM of c-Pc was observed. The Figure 3 shows the significant ($p<0.05$) optical density reduction (referred to CD formation at 245 nm) in the c-Pc-treated samples compared to the controls. The effects of c-Pc (40 µM) in the lipid oxidation resistance assay showed no statistical differences ($p>0.05$) as compared with Trolox-C, which is a recognized lipid phase soluble-antioxidant standard. C-Pc and Trolox were able to significantly delay ($p>0.05$) the time taken for CD to reach a maximum generation value as compared to the non-treated samples.

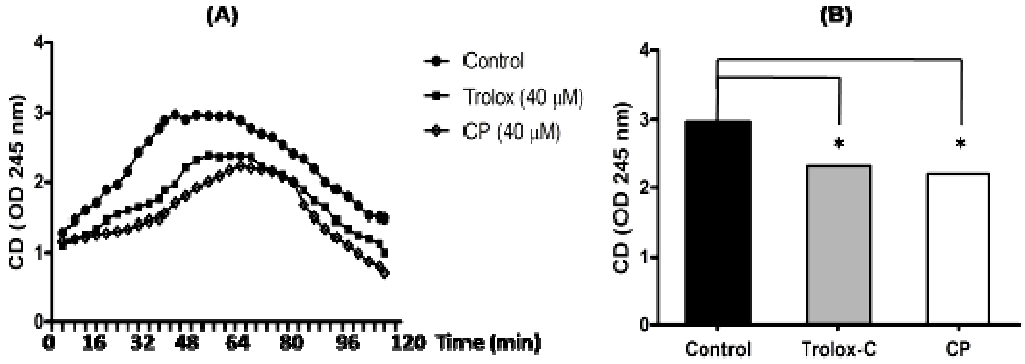

Figure 3. Kinetics of CD formation. Footnote: Inhibitory effect of c-Pc on CD formation. Kinetics was monitored every 4 minutes, during 2 hours. CP: samples treated with c-phycocyanin. OD: optical density

(corresponding to CD formation). Asterisks represent statistical differences ($p<0.05$) respect to the control samples.

Discussion

Different LPO products are formed from polyunsaturated fatty acids as a consequence of ROS action on lipoproteins or biomembranes. Utilized as biomarkers of lipid damage, MDA, 4-HA, lipoperoxides and CD have been extensively studied and represent a useful tool for redox studies [10]. The susceptibility to LPO of hepatic microsomes, measured by the MDA levels, showed that c-Pc protected the lipids against the ferric/ascorbic acid-induced oxidative insult. Various authors have been reported that c-Pc has potent free radical-scavenging properties [23-27], an important characteristic of c-Pc which may allow it to block LPO. Also, our data are in accordance with the results of [28], which found that c-Pc inhibited the LPO of hepatic microsomes induced by ferric/ascorbic acid. But in our study we also showed that c-Pc inhibited the formation of others LPO-derived products, such as lipoperoxides and 4-HA. Among them, the α,β-unsaturated aldehydes, such as 4-hydroxy-2-nonenal are highly reactive and readily react with phospholipids, proteins, and DNA to cause deleterious effects on cells [29].

On the other hand, lipid hydroperoxides are the first derived products from LPO [2]. Lipid hydroperoxides in the presence or absence of catalytic metal ions also give rise to a large variety of products, including short chain aldehydes, which can be used to assess the degree of LPO in a system [10]. Many natural products are able to inhibit the lipoperoxides formation [7]; indeed c-Pc inhibited the in vitro formation of TH as well as Trolox-C. The LPO system used by our group is based on Fenton reaction, this type of lipid oxidative damage is caused by metal ion (Fe^{2+}) with traces of hydroperoxides, forming an alkoxyl radical. The alkoxyl radical in turn propagates the free radical chain reaction. Considering these facts, our data suggest that c-Pc may inhibit this process at its earliest stage by removing the alkoxyl radicals or by binding the metal ions.

Plasma lipoproteins play an important role in the metabolism of cholesterol, and also have anti-and pro-atherogenic properties. Under oxidative stress conditions, the antioxidant, antiinflammatory and antiatherogenic properties of high density lipoproteins (HDL) are delayed [30], while oxidized low density lipoprotein (ox-LDL) have a potent atherosclerotic effects [31]. Thus, the capacity of natural compounds to block the lipoproteins oxidation, such as c-Pc, represents an attractive tool to treat many cardiovascular disorders. Indeed, c-Pc has been used in the treatment of atherosclerosis [32,33] and ischemia-reperfusion of the heart [34] with promissory results. On the other hand, the protection of lipids of cellular membranes by c-Pc (reinforced in the present work) may be responsible of its satisfactory use in the treatment of multiple sclerosis [35] and ischemia/reperfusion-induced brain injury [36].

Conclusions

Our data demonstrated the protective effect of c-Pc on lipoprotein against LPO and further reinforce the antioxidant properties of this biliprotein. The present work highlights the capacity of c-Pc to act as a powerful lipid-phase antioxidant. The positive results and the beneficial effects observed for c-Pc in many pathological disorders may be associated with the capacity to inhibit the LPO phenomenon, reinforced by our experimental findings.

Acknowledgement

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Conflict of interest

The authors declare that they have no conflict of interest.

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