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Antioxidant activity of different fractions of *Spirulina platensis* protean extract☆

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Abstract

Spirulina platensis, planktonic blue–green algae, is gaining increasing attention because of its nutritional and medicinal properties. This microalgae contains phycobiliproteins (phycocyanin and allophycocyanin). Previous reports from our laboratory have shown that a protean extract of *S. platensis* is a potent free-radical scavenger (hydroxyl and peroxyl radicals) and inhibits microsomal lipid peroxidation. The aim of this study was to purify and characterize phycocyanin of *S. platensis*. Besides, we tried to demonstrate that one of the main components responsible for this antioxidant activity is a biliprotein phycocyanin. For this purpose, we studied the antioxidant activity of different fractions obtained during the phycocyanin purification process, through the scavenger activity of hydroxyl radical. We also observed that an increase in phycocyanin content was related to an increase in the antioxidant activity in different fractions, and therefore phycobiliprotein phycocyanin is the component mainly responsible for the antioxidant activity. © 2001 Éditions scientifiques et médicales Elsevier SAS

Keywords: *Spirulina platensis*; Phycocyanin; Antioxidant; Protean extract

1. Introduction

It is now well recognized that an increased formation of oxygen radicals and other oxygen derivatives frequently accompanies tissue damage. Recently, there has been an explosive interest in the use of antioxidant nutritional supplements. Epidemiological evidence suggests that intake of some vitamins, minerals, and other food constituents may help to protect against heart disease, cancer and the aging process, and that antioxidants may have a protective effect, either in preventing these diseases or lessening the severity of the diseases upon their onset. Many of their activities are mediated by reactive oxygen species (ROS), which are generated during the oxidative burst [1–4].

Therefore, *Spirulina* is a traditional food of some Mexican and African people. It is a planktonic blue–green algae found in alkaline water of volcanic lakes. *Spirulina* has a 62% amino acid content and is the world's richest natural source of vitamin B_{12} and contains a whole spectrum of natural mixed carotene and xanthophyll phytopigments. *Spirulina* has a soft cell wall made of complex sugars and protein. Actually it is gaining more attention because of its nutritional and various medicinal properties.

Several studies show that *Spirulina* or its extracts can prevent or inhibit cancer in humans and animals. In vitro studies suggest that polysaccharides of *Spirulina* enhance cell nucleus enzyme activity and DNA repair synthesis. Besides, *Spirulina* is a powerful tonic for the immune system. Feeding studies show that even small amounts of *Spirulina* build up both the humoral and cellular mechanisms of the immune system [5–8].

Spirulina platensis also contains phycobilisomes as light-harvesting protein–pigment complexes. Phycobilisomes are mainly (80–85%) composed of brilliantly colored polypeptides named phycobiliproteins [9–12]. The two more important biliproteins in this microalgae are phycocyanin and allophycocyanin, both having the same chromophoric group [13,14].

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The aim of this study was to purify and characterize phycocyanin of *S. platensis*. Besides, we wanted to demonstrate that one of the components mainly responsible for this antioxidant activity is the biliprotein phycocyanin. For this purpose, we analyzed the antioxidant activity of different fractions obtained in the purification process of phycocyanin, through the scavenger activity of the hydroxyl radical.

2. Materials and methods

2.1.1. Protean extract preparation

S. platensis (1 g) (powder provided by GENIX company, Cuba) was mixed with distilled water (25 ml) for 24 h at 4°C. The crude phycocyanin obtained was centrifuged and the supernatant was precipitated with 50% ammonium sulfate and centrifuged again. The supernatant was discarded and the blue precipitate was dissolved in distilled water, dialyzed and lyophilized.

2.1.2. Purification of phycocyanin

The lyophilized aqueous extract was dissolved in 2.5 mM phosphate buffer pH 7.0 and applied to a Bio-Gel P gel (Bio-Rad) with exclusion limit between 1500 and 20 000 Da. Three fractions were obtained, one with a molecular weight higher than 20 000 Da (fraction 1), and two others (fractions 2 and 3) with lower molecular weight.

Fraction 1 was then placed onto a hydroxyapatite column (Bio-Rad) and eluted with phosphate buffer of increasing ionic strength between 2.5 and 100 mM. The fraction with the highest $A_{620/280}$ ratio was eluted between 50 and 70 mM at 0.5 ml/min of flow rate [13].

This fraction (supposed to contain phycocyanin, fraction 4) had to be further purified by DEAE Sephadex A-50 (Sigma) chromatography by a gradient of 0.15–0.45 M NaCl in 50 mM phosphate buffer pH 8.0 and a flow rate of 0.5 ml/min [15]. Absorbance ratio 620/650 was used as a criterion of purity [9,10,13].

Table 1
Absorbance ratios of the fractions obtained from the Bio-Gel filtration column and protean extract

	$A_{620/280}$ (phycocyanin)	$A_{650/280}$ (allophycocyanin)
Protean extract	0.520 ± 0.0024	0.303 ± 0.0018
Fraction 1	0.900 ± 0.0210 *	0.410 ± 0.0035*
Fraction 2		
Fraction 3		

* Significant differences in comparison to protean extract.

A 12% polyacrylamide gel electrophoresis (SDS-PAGE) of all fractions obtained in the purification process was carried out. Protein (10 μg) was dissolved in a sample buffer (60 mM Tris–HCl buffer pH 7.0) which contained 2% SDS, 14.4 mM β-mercaptoethanol, 25% glycerol and 0.1% bromophenol blue. This mixture was boiled for 5 min. Electrophoresis was run at 100 V for 1.5 h. The gel was stained with Coomassie Brilliant blue. The mobility of phycocyanin subunits was determined by reference to standard protein markers of known molecular weight. The phycocyanin used as patron was supplied by Europe-Bioproducts Ltd.

2.1.3. Scavenger activity on hydroxyl radical generated by the ascorbate/iron/H_2O_2 system

The reaction mixture contained the following reagents to the stated final concentrations: 2.8 mM deoxyribose; 20 mM phosphate buffer pH 7.4; different fractions of *S. platensis* at concentration 200 μg protein/ml in 20 mM phosphate buffer pH 7.4 (or phosphate buffer for control reactions); 100 μM $FeCl_3$; 104 μM EDTA; 1 mM H_2O_2; and 100 μM ascorbate. Solutions of $FeCl_3$ and ascorbate were made up immediately before use in deareated water. The reaction mixture was incubated at 37°C for 30 min. [16,17]. The extent of deoxyribose degradation, by the hydroxyl radicals formed, was measured by using the thiobarbituric acid test (TBA test) [18].

2.2. Statistics

Data are presented as the mean ± SEM. Results are expressed as mean of percentage inhibition ± standard error of the mean (SEM). Student's *t*-test was used for comparison between means. The difference was considered statistically significant when $P < 0.05$.

3. Results

As shown in Table 1, protean extract from the microalgae *S. platensis* is separated in a Bio-Gel filtration column with exclusion limit between 1500 and 20 000 Da, into three fractions. Fraction 1 showed a $A_{620/280}$ ratio (representative measure of the quantity of phycocyanin) significantly higher than the protean extract. This first fraction contained substances with a molecular weight higher than 20 000 Da. Fractions 2 and 3 presented a ratio close to zero, which is an indication of the absence of phycocyanin.

The purification steps are summarized in Table 2. Fraction 1 was transferred to a hydroxyapatite column, equilibrated with 2.5 mM phosphate buffer, pH 7. Elution was performed with phosphate buffer of increasing ionic strength from 2.5 to 100 mM. The fraction with the higher $A_{620/280}$ ratio (fraction 4) was eluted

Table 2
Data from the purification of the phycocyanin obtained from the blue–green alga *Spirulina platensis*

Purification step	$A_{620/280}$ (phycocyanin)	$A_{650/280}$ (allophycocyanin)
Protean extract	0.520 ± 0.0024	0.303 ± 0.0018
Bio-Gel filtration column	0.900 ± 0.0210 *	0.410 ± 0.0035*
Hydroxyapatite column	2.160 ± 0.1800 *	0.980 ± 0.0360*
DEAE Sephadex A-50 column	3.900 ± 0.2000 *	0.514 ± 0.0632*

* Significant differences in comparison to protean extract.

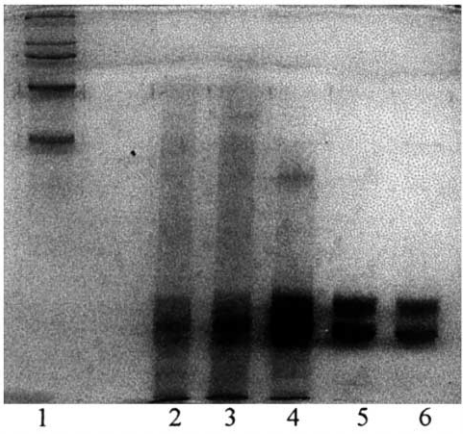

Fig. 1. SDS-PAGE (1 — high molecular weight marker; 2 — protean extract; 3 — fraction 1; 4 — fraction 4; 5 — phycocyanin obtained, standard phycocyanin).

between 50 and 70 mM and the absorbance ratios 620/280 were 2.160 ± 0.1800 and 650/280 0.980 ± 0.0360 for phycocyanin and allophycocyanin, respectively. These values were significantly higher than those of fraction 1.

The complete purification of phycocyanin was achieved by ion exchange chromatography on DEAE-Sephadex A-50 from fraction 4. The column was eluted with 0.15–0.45 M NaCl gradient in 50 mM phosphate buffer, pH 8.0. Phycocyanin was eluted between 0.35 and 0.45 M and exhibited an absorbance ratio of $620/280 = 3.900 \pm 0.200$.

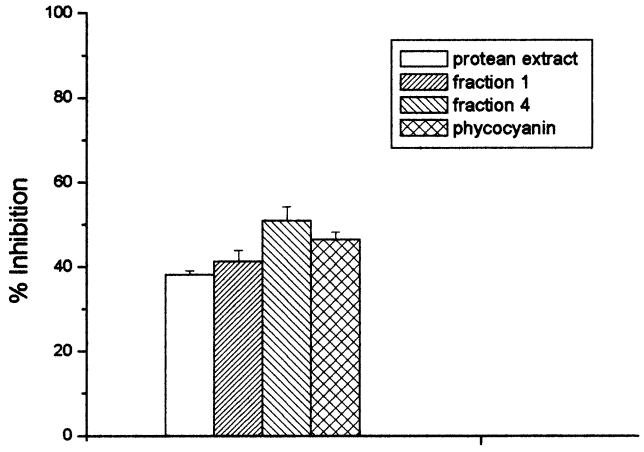

Fig. 2. Percentage inhibition of different fraction obtained from the protean extract during the purification process of phycocyanin.

To corroborate these results, the fractions obtained in the different purification steps of phycocyanin were subjected to gel electrophoresis SDS-PAGE (Fig. 1).

At the same time we have studied the hydroxyl radical scavenger activity of the protean extract as well as of the fractions obtained in the different purification steps of phycocyanin. The results are shown in Table 3. The relation between the inhibition values of the hydroxyl-generated damage, obtained from different fractions and their content of Phycocyanin is indicated in Fig. 2.

4. Discussion

The results achieved in the purification process of phycocyanin corroborate the efficiency of the methods employed. The fractions obtained during this process showed an absorbance ratio 620/280 higher than the protean extract. The ratio for phycocyanin at the end of the process was approximately 4, the reported value for pure phycocyanin [13].

Moreover, through SDS-PAGE (Fig. 1) we observed that after purifying our extract, some bands (that correspond to other proteins) disappeared and there was an increase in the characteristic phycocyanin pure band.

Table 3
Scavenging effects of protean extract and fractions obtained in the purification process of phycocyanin on hydroxyl radical production generated by the ascorbate/iron/H_2O_2 system

	$A_{620/280}$ (phycocyanin)	$A_{650/280}$ (allophycocyanin)	Percentage inhibition
Protean extract	0.520 ± 0.0024	0.303 ± 0.0018	38.12 ± 0.90
Fraction 1	0.900 ± 0.0210	0.410 ± 0.0035	41.20 ± 2.68
Fraction 2			15.00 ± 1.63
Fraction 3			14.35 ± 2.04
Fraction 4	2.160 ± 0.1800	0.980 ± 0.0360	50.90 ± 3.25
Phycocyanin	3.900 ± 0.2000	0.514 ± 0.0632	46.40 ± 1.86

This was confirmed by comparing our purified phycocyanin and the standard product.

Fractions 2 and 3 obtained from the protean extract, which were not containing biliproteins, showed lower antioxidant activity than fraction 1 that contains phycocyanin (statistically significant). At the same time, fraction 4, rich in phycocyanin, showed significantly stronger activity than fraction 1, which is related to the higher content of this biliprotein (Table 3).

The inhibition values in the radical hydroxyl from the different fractions, and their related increase in the phycocyanin content (Fig. 2) demonstrate the antioxidant activity of this biliprotein. Furthermore, biliprotein was proved to be mainly associated with the antioxidant activity of the protean extract of *S. platensis*.

In the case of the pure phycocyanin, one would have to expect an increase in the percentage inhibition as compared to fraction 4. However, we did not observe statistically significant differences between the antioxidant activities shown by the both samples. Maybe these differences could be attributed to the absence of allophycocyanin a biliprotein that apparently also manifests antioxidant properties.

In conclusion, increase in the amount of phycocyanin is related to the increase in the antioxidant activity in the different fractions, and therefore phycobiliprotein phycocyanin is the component mainly responsible for the antioxidant activity of the protean extract of *S. platensis*.

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