Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2013, 5(5):332-336

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

An *in vitro* study of c-phycocyanin activity on protection of DNA and human erythrocyte membrane from oxidative damage

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ABSTRACT

Biological macromolecules such as proteins and nucleic acids are highly prone to oxidative damage leading to a pathogenesis of many diseases. Previous studies have shown that c-phycocyanin (c-PC), a pigment found in cyanobacteria, has scavenging activities on various free radicals. In the present study, c-PC from Spirulina platensis was investigated for its protective activity against oxidative damage on plasmid DNA and erythrocyte membrane proteins. The results showed that c-PC can reduce percentages of DNA damage induced by Fenton's reaction in a dose dependent manner. C-PC also possessed a protective effect on erythrocytes membrane protein. As demonstrated by SDS-PAGE analysis of red blood cells membrane, c-PC can prevent the degradation of red blood cell skeleton upon exposure to hydrogen peroxide. Both experiments showed that antioxidative activity of c-PC is higher than that of Trolox at the same concentration. The results suggested that c-phycocyanin may offer new therapeutic approaches for oxidative stress-mediated diseases.

Keywords: antioxidant, DNA, erythrocyte membrane, oxidative damage, phycocyanin

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INTRODUCTION

In living organism, reactive oxygen species (ROS) were generated during normal cellular metabolism. High concentration of ROS leads to damage on macromolecules in living cells such as DNA, proteins, and lipids [1]. The presence of free radicals which exceeds the capacity of antioxidant defense system is called "oxidative stress" [2]. The oxidative stress has been found to associate with pathological conditions in several degenerative diseases such as Parkinson's disease, Alzheimer's disease, artherosclerosis, Rheumatoid arthritis, malaria, and cancer [1-3]. Many natural products have been studies in order to search for potential antioxidant activities [4-5].

Spirulina spp. is well known as a source of nutrients and therapeutic values such as anticancer [6], radioprotection [7], antiviral [8], immunomodulatory properties [9], and antioxidant properties [10]. It has also been used as adatoagen for reduction of oxidative stress caused by bioaccumulation [11]. One of major pigment constituents of *Spirulina* spp., c-phycocyanin (c-PC), has been recognized as a potent antioxidant compound [12-14]. C-PC is a blue-coloured protein in a phycobiliprotein family which consists of two subunits; α- and β-subunit. Each subunit contains apo-protein covalently bond to at least one tetrapyrrole chromophore (phycocyanobilin; PCB) which seems to involve in its antioxidant activity [15]. C-PC was previously reported to provide several pharmaceutical properties such as anti-inflammatory [16], anti-cancer [17], and anti-platelet aggregation [18].

Excess ROS, especially H_2O_2, can cause harmful effects on DNA damage attributed to DNA mutation and cancer [19, 20]. It was previously reported that hydroxyl radical, a strongly toxic oxidant derived from superoxide radicals and hydrogen peroxide, is involved in DNA damage by converting guanine into 8-hydroxyguanine [21]. Another target of free radicals is erythrocytes. Erythrocytes are prone to oxidative damage due to their high contents of polyunsaturated fatty acids (PUFA) at the membrane, high intracellular oxygen concentration, and high level of hemoglobin carrying iron. These components are considered as potential ROS promoters [22]. Within erythrocytes, oxidative damage was started in lipid membrane by peroxidation lead to increase of membrane viscosity [23]. Such effects result in short life-span of erythrocytes. Therefore, protection of oxidative damage to these two important biomolecules would prevent or reduce pathological conditions of various oxidative stress-related diseases.

This study aims to investigate radical scavenging activity of c-PC on protection of DNA damage induced by Fenton's reaction and to study their protective effects on human erythrocyte membrane damage induced by H_2O_2 compared to Trolox, a water soluble derivative of vitamin E.

EXPERIMENTAL SECTION

C-phycocyanin extraction and purification

C-phycocyanin (c-PC) was extracted and purified according to Boussiba *et al*. [24] with some modifications. C-PC was prepared from approximately 10 g of wet *Spirulina platensis* (Taweewattana Farm, Bangkok, THA) by salt precipitation with 35% and 50% ammonium sulphate (Bio-Rad, Canada, USA). The precipitate from 50% ammonium sulphate which contained c-PC was dissolved in 0.025 M sodium phosphate buffer (pH 7.0). After dialysis with the same buffer, the dialyzed sample was applied to a DEAE-Sepharose Fast Flow column (GE Healthcare, Uppsala, SE) size 2.5 x 30 cm. The purification process was performed according to the manufacturer's recommendations. The protein was eluted with a linear increasing concentration (0-0.25 M) of NaCl in Tris-HCl buffer (pH 7.4) at a flow rate of 1 ml/minute and the 2 ml fractions were collected. The fractions with ratio of absorbance at 620 nm and 280 nm (A620/280) more than 4.0 were pooled and further processed as purified c-PC. The pooled fractions were desalted by dialysis with 0.025 M sodium phosphate buffer (pH 7.4). The dialyzed purified c-PC was concentrated and protein concentration was measured using Bio-rad protein assay (Bradford) [25]

Effect of c-PC on protection of DNA oxidative damage

DNA damage protection assay was conducted according to Chen et al. Chen *et al*. [26] by using the Fenton's reagent with some modifications. Plasmid pUC18 (Promega, Madison, USA) was multiplied in E. coli (DH5α) and extracted using plasmid extraction kit (Macherey-Nagel, Dueren, DE). The reaction mixtures composed of 250 ng pUC18, varying concentrations (2.5, 5, 10, and 20 μM) of c-PC or Trolox (Sigma, St. Louis, USA)). The total volume was adjusted to 15 μL with 50 mM PBS, pH 7.4. Then, $FeSO_4$ (Ajax Finechem, Sydney, AU) and H_2O_2 (Merck, Darmstadt, DEU) were added to the reaction mixtures to give the final concentration of 1 mM and 50 mM, respectively. After incubated at 37°C for 60 minutes, the reaction mixture was placed in 1% agarose gel for electrophoresis and run at 70 volts for 70 minutes. The DNA was visualized and photographed using Syngene gel documentation system (Syngene, Maryland, USA). DNA band quantification was performed using GeneTools software (Syngene, Maryland, USA).

Erythrocyte preparation

Blood samples were obtained from healthy volunteers by venipuncture, and collected into heparin anti-coagulation tube. The erythrocytes were separated by centrifugation at 2,500 rpm, 4°C for 10 minutes. Plasma and buffy coat were removed. The erythrocytes were washed three times with PBS (27 mM Na2HPO4, 123 mM NaH2PO4 and 123 mM NaCl, pH 7.4) and resuspended in PBS to obtain erythrocytes suspension at 10% hematocrit.

Effect of c-PC on protection of human erythrocyte membrane from H_2O_2

The erythrocyte oxidative damage was performed using H_2O_2 as a free radical initiator. The reaction mixtures contained 5% suspension of erythrocytes in PBS, c-PC or Trolox with different concentrations (0.1 and 0.5 μM), and H_2O_2 at a final concentration of 300 mM. The mixtures were incubated by shaking gently for 120 minutes at 37°C. The negative control containing only erythrocytes suspended in PBS and the positive control containing erythrocytes and H_2O_2 were included. After incubation, erythrocyte membranes were prepared according to Fairbanks *et al.* [27] using 5 mM phosphate buffer (PB, pH 8.0) as a hypotonic lysis buffer. The protein concentration was measured according to the method of Bradford [25] (Bio-Rad, Canada, USA). The erythrocyte membrane proteins were analysed by 7.5% discontinuous SDS-PAGE according to the method of Leammli [28] using 20 μg of membrane proteins. The protein bands were stained with Coomassie brilliant blue (Bio-Rad, Canada, USA).

RESULTS AND DISCUSSION

Effect of c-PC on protection of DNA oxidative damage

Damage of plasmid DNA, pUC18, could be monitored by agarose gel electrophoresis. The intensity of DNA band at the position of linear form (nicked plasmid) along with the decrease of DNA band in the position of supercoiled from (intact plasmic) represented the amount of DNA damage (figure 1). The band intensity was estimated by gel documentation system. The percentage of DNA damage was calculated from the ratio of linear DNA band intensity and the total DNA band intensity (figure 2). The result showed that intact pUC18 presented mostly in supercoiled form. The intensity of supercoiled DNA and linear DNA were 76% and 24%, respectively. Exposure of pUC18 $H_2O_2/FeSO_4$ resulted in increase of linear plasmid to 57%. The percentage of DNA damage was reduced by addition of c-PC or Trolox. For c-PC at 2.5, 5, 10, and 20 µM, the percentage of DNA damage was 34%, 30%, 26%, and 25%, respectively. For Trolox at 2.5, 5, 10, and 20 µM, the percentage of DNA damage was 67%, 55%, 50%, and 47%, respectively. These results indicated that, at the same concentrations, c-PC was more effective than Trolox in protection of DNA from oxidative damage induced by Fenton's reaction. Besides, at a concentration of 2.5 µM Trolox, the percentage of DNA damage was increased from the positive control. There are several evidences [1, 29-31] show that excess ROS leads to oxidative damage of biomolecules such as DNA, lipids, proteins, and cell membrane. Recently, the DNA damage has been reported to be implicated in many human diseases [32]. In this study, we investigated the protective effects of c-PC as an inhibitor for DNA damage reaction induced by hydroxyl radicals generated from Fenton's reaction. The effect of c-PC on scavenging of hydroxyl radicals may be due to its direct chemical neutralization based mainly on the scavenging activity of pycocyanobilin (PCB). As demonstrated by Bhat and colleagues, the oxidizing property of peroxinitrite ($ONOO^-$) could be inhibited by 2.9 µM of pycocyanobilin (PCB) resulted in prevention of plasmid pBR322 damage [33]. C-PC exhibit higher antioxidant potential than trolox which might be attributed to its high content of active component. C-PC consists of tetrapyrrole chromophores (PCB) covalently bonds with apo-protein [34], while Trolox structure is called chroman head consisting of one phenolic and one heterocyclic ring [35]. The antioxidant properties of such active compounds were initiated by hydrogen donation from the hydroxyl group to radicals [35]. Thus, the hydrogen donation of c-PC is from at least four pyrroles group for an alpha subunit and eight pyrroles group for a beta subunit, whereas hydrogen donation of trolox is from one phenolic group.

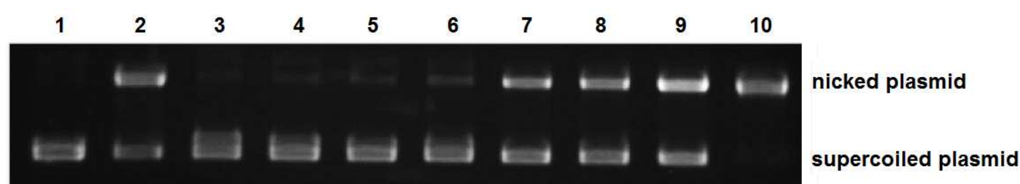

Figure 1- Plasmid pUC18 electrophoris showing protective effects of c-PC or trolox against oxidative damage; Lane 1 pUC18 (untreated), lane 2 pUC18 treated with H_2O_2/FeSo$_4$, lane 3-6 pUC18 treated with H_2O_2 and 20,10, 5, and 2.5 µM c-PC, respectively, lane 7-10 pUC18 treated with H_2O_2/FeSo$_4$ and 20,10, 5, 2.5 µM Trolox, respectively. The upper row represents the linear form of damaged plasmid and the lower row represents the intact plasmid presented in supercoiled form.

Effect of c-PC on protection of human erythrocyte membrane from H_2O_2

The alterations in erythrocyte membranes protein induced by H_2O_2 and the capability of c-PC on membrane protection were investigated by SDS-PAGE. Exposure of erythrocyte to high concentration of H_2O_2 led to the degradation of all of major membrane proteins as shown in figure 3, lane 1. In the presence of either c-PC or Trolox (0.1 and 0.5 µM), bands of major membrane proteins could be observed (figure 3, lanes 3-7) at the same intensity as the intact erythrocytes (figure 3, lanes 2). We could not reveal the difference between c-PC and Trolox and the difference between the two concentrations. Human erythrocyte is a good model for studying about oxidative damage due to its susceptibility towards oxidative stress induced by lipid peroxidation products. Moreover, oxidative hemolysis plays important role in many hematologic diseases. It has been reported that proteolytic enzymes in erythrocytes could be activated by different oxygen radicals [36]. Ajila *et al* found that membrane proteins were degraded when treated erythrocytes with H_2O_2 [37]. The results obtained from this study indicate that c-PC can protect erythrocyte membrane protein from degradation induced by oxidative damage which in turn preventing hemolysis. Anti-hemolytic effect of c-PC has been revealed by Romay and Gonzalez [38]. They demonstrated that c-PC has ability to inhibit peroxyl radicals-induced erythrocyte hemolysis due to its scavenging effect of radicals in aqueous phase.

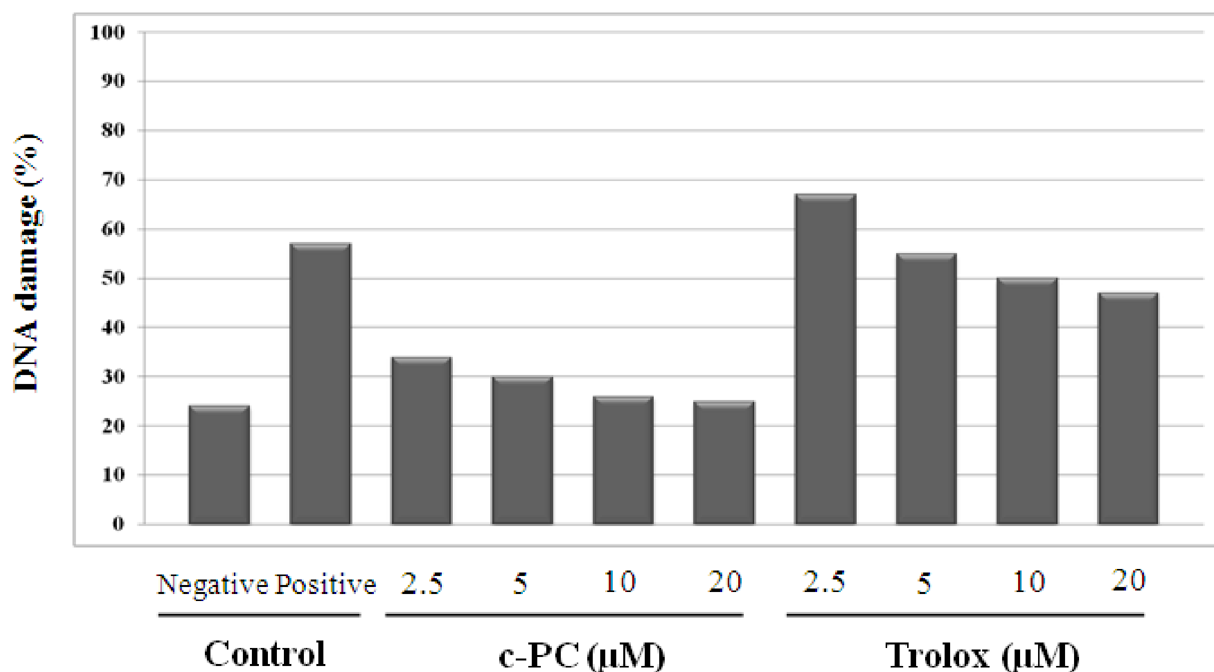

Figure 2-The percentages of DNA damage of plasmid pUC18 treated with $H_2O_2/FeSo_4$ in the presence of different concentrations of c-PC or Trolox. The negative control containing only pUC18 in the reaction buffer and the positive control containing pUC18 and $H_2O_2/FeSo_4$ were included.

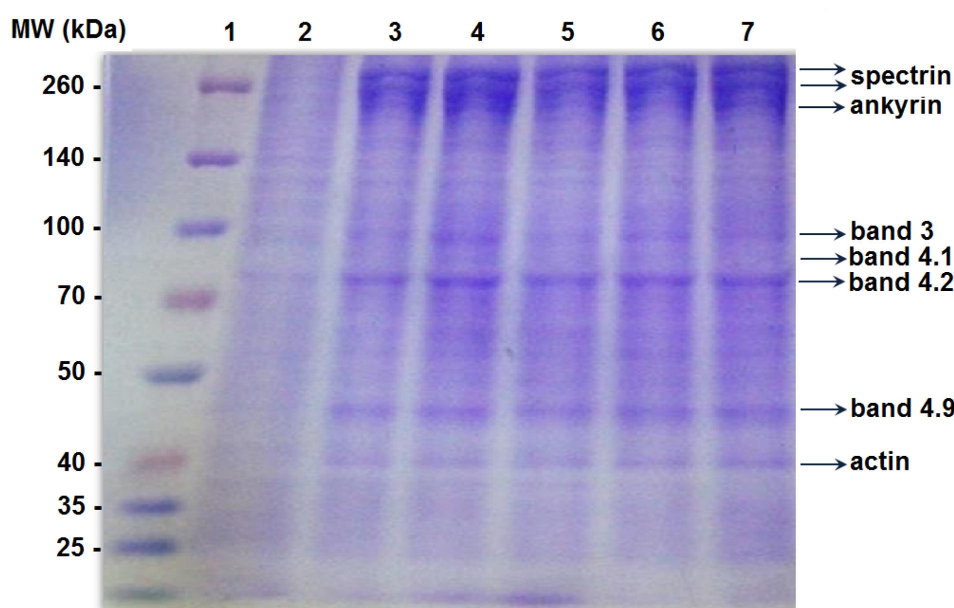

Figure 3-SDS-PAGE analysis showing pattern of erythrocyte membrane proteins upon exposure to H_2O_2 with or without antioxidant compounds; Lane 1 Molecular weight protein markers, lane 2 erythrocyte membrane proteins treated with H_2O_2, lane 3 erythrocyte membrane proteins (untreated), lane 4-5 erythrocyte membrane proteins treated with H_2O_2 in the presence of 0.1 and 0.5 µM c-PC, respectively, lane 6-7 erythrocyte membrane proteins treated with H_2O_2 in the presence of 0.1 and 0.5 µM Trolox, respectively.

CONCLUSION

In this study, antioxidant activity of c-PC from *Spirulina platensis* have been evaluated using different models; plasmid DNA and erythrocyte membrane. C-phycocyanin was proved to be a potent antioxidant which can protect DNA and membrane proteins from oxidative damage. The results suggested that c-phycocyanin may be used as a supplement for therapeutic approach in oxidative stress-related diseases. Further studies should be performed to provide more understandings about antioxidant mechanism *in vivo*

Acknowledgements
This study was supported by Chulalongkorn University graduate scholarship from Ratchadaphiseksomphot Endowment Fund on the occasion of the 90th anniversary of Chulalongkorn University.

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