Role of β-Cryptoxanthin as an Antioxidant and Its Ability to Bind with Transferrin

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Abstract: The potential nutraceutical benefits of carotenoids, which are versatile bioactive compounds, have been of great interest recently for its applications as dietary supplements. Carotenoids are red-orange coloured pigments which absorb light in the wavelength region of 400-550 nm. They are produced by plants, bacteria, algae and fungi and are abundantly distributed in the nature. In this study, our focus is on beta-cryptoxanthin (β-CRX), a yellow colored pro-vitamin A xanthophyll which is extracted from a previously isolated bacterium *Kocuria marina* DAGII grown in Brain Heart Infusion and sub-cultured in low cost dairy waste like whey and incubated at 25°C and 150 rpm for 5 days. The extracted beta-cryptoxanthin showed good radical scavenging activity and played a role in inhibition of lipid oxidation.

Transferrin is a glycoprotein which plays a significant role in the mobilisation of iron in the body. It has two receptors TfR1 and TfR2 amongst which TfR1 binds to the iron-loaded transferrin. In cases of secondary hemochromatosis, HFE protein competes with transferrin to bind to TfR1 which leads to iron built up which is detrimental to the human body. Beta-cryptoxanthin was found to bind to Transferrin with a binding energy of -8.2 kcal/mol.

Key words: Beta-cryptoxanthin, lipid oxidation, radical scavenging, transferrin.

1. Introduction

Free radicals, antioxidants and co-factors are the three main areas that contribute to the delay in the aging process. The understanding of these events in the human body can help prevent or reduce the incidence of diseases, improving the quality of life. Reactive Oxygen Species' (ROS) effects on cells include not only roles in apoptosis (programmed cell death) but also positive effects, such as the induction of host defense genes, the stimulation of the adaptive immune system via the recruitment of leukocytes, and mobilization of ionic transport systems in the so-called redox or oxidative signaling. Oxidative stress caused by the imbalance between ROS and biological antioxidant systems can lead to modification of these macromolecules subsequently, in the case of excessive amounts, ROS can determine deleterious effects. Free radicals play a crucial role in the progression of many pathologies, such as atherosclerotic processes, myocardial and cerebral ischemia, renal failure, asthma, Parkinson's disease, kidney damage, preeclampsia, and more general inflammation, as well as all the chronic degenerative diseases.

Normally, cells defend themselves against ROS damage through intracellular and extracellular defenses, in particular through enzymes. Exogenous antioxidants such as ascorbic acid (vitamin C), carotenoids, tocopherol (vitamin E), and polyphenols also play important roles in preventing ROS damage by scavenging free radicals. Carotenoids are components that play an important role in biological systems, starting with

light protection, immunoenhancement, protection against carcinogens and finishing with antioxidant activity [1], [2]. Moreover, aside from the provitamin A activity, these compounds have health-promoting effects: immunoenhancement and reduction of the risk of developing degenerative diseases such as cancer, cardiovascular diseases, cataract and macular degeneration.

One such carotenoid is β-CRX, a xanthophyll, valued for the pleasing yellow, orange or red color they impart to many foods and has displayed risk reduction in both epidemiological studies and supplementation human trials [3]. This conjugated double-bond structure makes carotenoids strong scavengers of singlet oxygen. They act via electron acceptance, physical quenching donation or via hydrogen abstraction/acceptance. β-CRX not only scavenges ROS but also prevents protein, lipid, or DNA oxidative damage by regulating other antioxidant mechanisms. It's also been found that high levels of antioxidants significantly decreases age-related macular degeneration (AMD) progression and vision loss risk [4]. In this study β-cryptoxanthin extracted from *Kocuria marina* DAGII (Accesion No. KF498648) is investigated to evaluate its potential to scavenge free radicals and help in the inhibition of lipid oxidation.

Iron deficiency and Vitamin-A deficiency anemias are public health problems in developing countries as it causes impairment of erythropoiesis and iron metabolism. Vitamin A supplementation mobilizes iron from existing stores in bone, liver, spleen etc to support increased erythropoiesis. It has been found that low plasma retinol levels are associated with low concentrations of haemoglobin and serum Fe and low degrees of transferrin saturation. In the human body, β-CRX is the only xanthophyll which is a precursor of vitamin A (retinol), an essential nutrient needed for eyesight, growth, development, and immune response, and is therefore considered as pro-vitamin A [5]-[7].

In this study we investigate the possible role of β-cryptoxanthin in combating anemia by studying its structure and possible interaction with Transferrin and Transferrin receptors .Transferrin receptor 1 (TfR) is a homodimeric type II membrane protein that plays a critical role in the primary iron acquisition mechanism [8]. TfR1 binds the serum iron-carrier protein transferrin (Fe-Tf). TfR also binds the hereditary hemochromatosis protein HFE, the protein mutated in patients with the iron overload disorder hereditary hemochromatosis. HFE and Fe-Tf can bind simultaneously to TfR1 to form a ternary complex, but HFE binding to TfR lowers the apparent affinity of the Transferrin - TfR1 interaction [9], [10]. The authors hypothesise that β-cryptoxanthin, binding to both Transferrin and the Transferrin Receptor(1&2) individually, might help in favouring the binding of Fe-Tf to TfR1 over HFE, thus helping in iron mobilisation and preventing iron built up in the cell. Although sufficient structural information is not available on HFE-TfR2 binding, and the consequent lowering of Transferrin-TfR2 affinity, the authors would like to propose that association of TfR2 with β-cryptoxanthin also favours the Transferrin-TfR2 binding through in silico docking studies.

2. Procedure and Materials

2.1. Microorganism, Cultivation Medium and Pigment Extraction

The microorganism *Kocuria marina* DAGII was isolated from soil during routine screening of pigment producing microorganisms in the department of Biotechnology, NIT Durgapur [11]. This bacteria produces β-cryptoxanthin (β-CRX). The strain was aerobically maintained in Brain Heart Infusion (BHI) agar slant at 4°C for regular use and is routinely sub cultured. Its accession number obtained is KF498648. Cultivation medium had a composition of Milk Whey (12.03% v/v), Yeast Extract (11.47gm/L), Peptone (5.29gm/L) and NaCl (4.0gm/L) and maintained at pH- 7.4. and was incubated at 25ºC, at 150 rpm for 120 hours. Then pigment extraction was done [12].

2.2. DPPH Antioxidant Assay

The antioxidant activities of β-CRX were determined by a method proposed by Li, Du, Jin, & Du, 2012 with slight modifications using the method of Samadarsi and Dutta (2018) [13]. A stock solution of DPPH (0.1 mM) was prepared in absolute ethanol and 0.1 ml of sample solution was added in 2.9 ml of DPPH stock solution. The reaction mixture was incubated in dark at room temperature for 30 mins, and the absorbance was measured at 517 nm. The control solution contained the same amount of buffer and DPPH radical.

2.3. Lipid Oxidation Measurements

The antioxidant activities of β-CRX were determined by a method as described in Garcia et al. 2005 [14]. 350µl of tissue homogenate is added to 500µl sample and we have a blank and empty control. To all three of them 250µl trichloroacetic acid at 40% was added and centrifuged at 3000 × g for 15 mins. All these operations were carried out at 4°C or on ice. 500µl of thiobarbituric acid reagent (0.67% TBA and 0.05 N NaOH in 50 ml of deionized water) was added to all three and then afterwards heated at 100°C for 15 mins. After heating, the tubes were cooled in a water bath at room temperature. Clear solutions were obtained with this procedure suitable for direct spectrophotometric measurement at 532 nm.

2.4. Structural Analysis

2.4.1. β-cryptoxanthin interaction with transferrin

Autodock software [15] was used to check the binding affinity of β-cryptoxanthin to the Transferrin protein. The structure of β-cryptoxanthin was obtained from PubChem(Id: 5281235) [16] and the Transferrin structure was obtained from PDB (Id:1D3K) [17].

2.4.2. β-cryptoxanthin interaction with transferrin receptor 1 and 2

Autodocking was done to check the binding affinity of β-cryptoxanthin to TfR1, the structure was obtained from PubChem(Id: 1SUV) and TfR2, the structure was obtained from UniProt (Id:QNUP52) [18].

3. Results and Discussions

3.1. Extraction of β-Cryptoxanthin

The extraction of β-cryptoxanthin from *K. marina* DAGII was carried out by two stage solvent extraction method. After extraction of β-cryptoxanthin in petroleum benzene, free radical scavenging assays were carried out with methanolic solvent.

3.2. DPPH Antioxidant Activity

Investigations in the radical scavenging activity of β-cryptoxanthin extracted from *K. marina* DAGI was done using the radical scavenging method. The DPPH scavenging activity was measured as percentage radical scavenging, using the formula:

$$\text{Scavenging Activity \%} = 100(1 - A_{sample}/A_{control}) \quad (1)$$

where, A_{sample} and $A_{control}$ represent the absorbance of the sample reaction and the absorbance of control reaction at 517 nm respectively.

Percent scavenging activity of β-cryptoxanthin was found to be 68%. A positive control using ascorbic acid was used and it was found to be 80%. A known bioactive compound with antioxidant activity, Mangiferin standard was taken as the positive control, which showed lower antioxidant activity as compared to extracted β-cryptoxanthin (The experiment was done in triplicates). The results are given in Fig. 1.

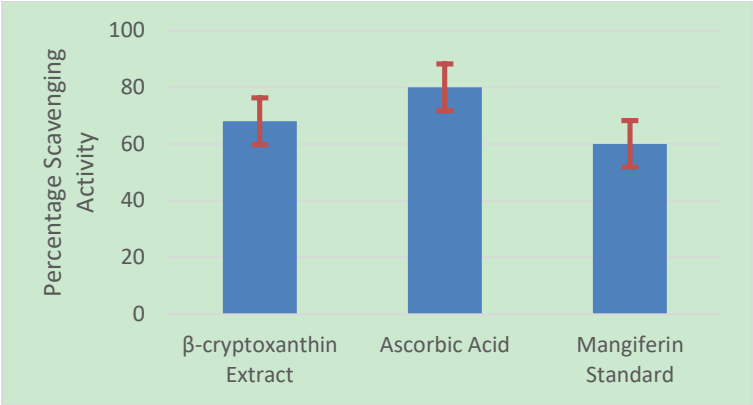


Fig. 1. Comparison of the SA percentages of β-cryptoxanthin extract, ascorbic acid and mangiferin standard.

3.3. Lipid Oxidation Measurement

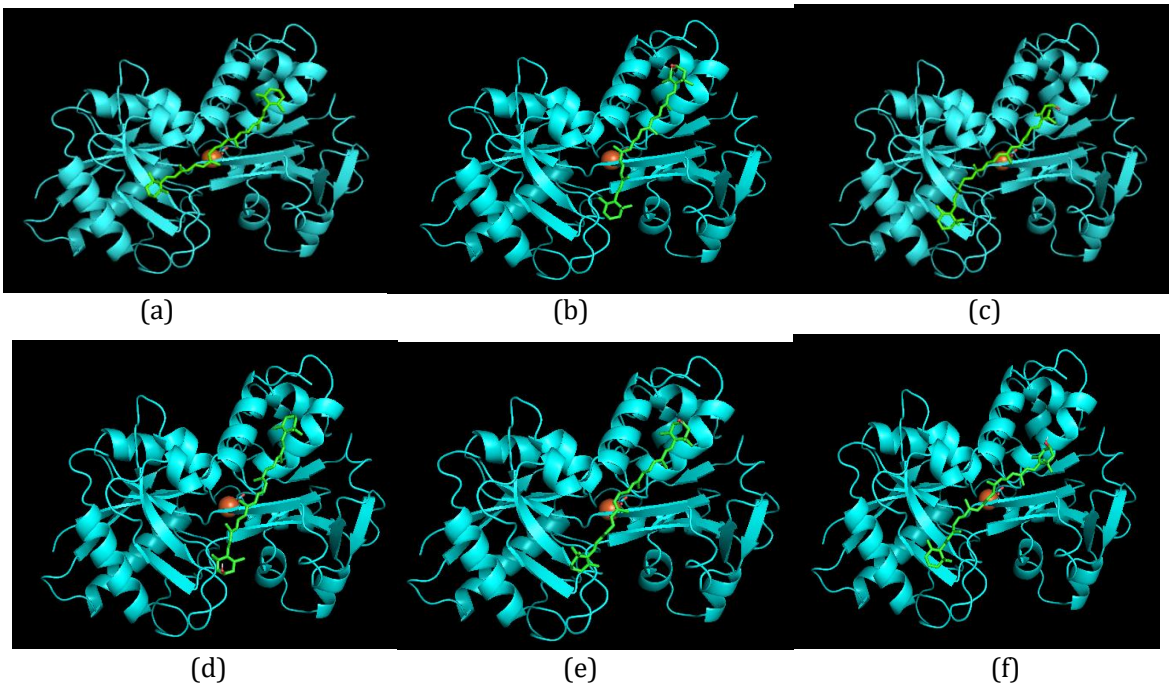
Lipid oxidation is a complex process initiated by free radical attack on the unsaturated fatty acid and is propagated by a lipid chain reaction cycle. β-CRX could inhibit the lipid oxidation and the percent inhibition of lipid oxidation was found to be 72% and it was calculated using the formula [19]. (The experiment was done in triplicates).

$$\text{Percentage Inhibition of lipid Oxidation} = 100 - [(A_{sample} - A_{control})\text{x}100] \quad (2)$$

3.4. Structural Analysis

3.4.1. β-cryptoxanthin interaction with transferrin

β-CRX was found to bind with Transferrin in 9 different orientations, with the best binding affinity being -8.2 kcal/mol. The 6 best orientations are given in Fig. 2. In all orientations, it was found that the β-CRX aromatic ring, bound to 3 amino acid residues, namely Gly-133, Leu-326, Val-246. The bonding maybe due to H-bonds and Van der Waal's bonds with the Transferrin protein.



(a) (b) (c)

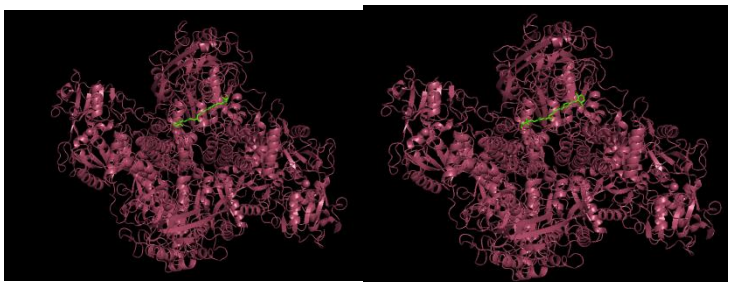
(d) (e) (f)

Fig. 2 (a). -8.2(kcal/mol); (b). -8.2(kcal/mol); (c). -7.8(kcal/mol); (d). -7.6(kcal/mol); (e). -7.3(kcal/mol); (f). -7.2(kcal/mol).

Fig. 2 Docking Images of β-CRX with Transferrin. The blue chain denotes the Transferrin molecule, whereas the green 3d chain seated deep inside the Transferrin molecule is the β-CRX, at the position where it is predicted to bind best. For all the 6(a-f) orientations, the binding affinities are mentioned below the images.

3.4.2. β-cryptoxanthin interaction with transferrin receptor 1

β-CRX was found to bind with TfR1 in 6 different orientations, with the best binding affinity being -7.3kcal/mol. The 2 best orientations are given in the Fig. 3. Thus, from this results, it can be hypothesised that the β-CRX bound transferrin will have better probability of binding over HFE. Maybe β-CRX molecule is acting as an intermediate, which increases the binding affinity of Transferrin to TfR1. For all the different orientations, it was found that the aromatic ring of β-CRX bound to 2 amino acid residues, namely Ala-546, Tyr-689. The bonding maybe due to H-bonds and Van der Waal's bonds with the TfR1 protein.



(a) (b)

Fig. 3 (a). -7.3(kcal/mol); (b). -6.9(kcal/mol).

Fig. 3 Docking Images of β-CRX with Transferrin Receptor 1. The red chain denotes the TfR1 molecule, whereas the green 3d chain seated deep inside the TfR1 molecule is the β-CRX, at the position where it is predicted to bind best. For all the 2(a-b) orientations, the binding affinities are mentioned below the images.

4. Conclusion

As per our objective we extracted β-cryptoxanthin from *K marina* DAG II by two stage solvent extraction procedure, using methanol and petroleum ether. We found that extracted β-cryptoxanthin had strong free-radical scavenging activity as seen from the DPPH assay and lipid oxidation assay which gave Percentage Scavenging activity of 68% and Percentage Inhibition of lipid oxidation of 72% respectively. This allows us to hypothesise that β-cryptoxanthin can be used as a nutraceutical because of its free radical scavenging activity and effective inhibition of lipid oxidation. In silico results also showed that β-cryptoxanthin has a very good binding affinity with both Transferrin and Transferrin Receptor 1, and thus might act as an intermediate helping the Transferrin bind to Transferrin Receptor 1. This opens up avenues for future research on β-cryptoxanthin playing an important role in mobilisation of iron and finding applications in the treatment of anemia.

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