Short Paper

Radical Scavenging Activity of Phycocyanobilin Prepared from the Cyanobacterium, *Spirulina platensis*

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Spirulina platensis contains phycocyanin as a light-harvesting protein for photosynthesis. Phycocyanin has an intense blue color since the open tetrapyrrole chromophore, phycocyanobilin, is covalently linked to the apoprotein. The blue protein has been extracted from the frond of *S. platensis* and widely used as a natural colorant for food additive to provide a blue color.¹⁾ The structure of phycocyanobilin is very similar to bilirubin and its catabolic precursor biliverdin. Bilirubin is known as a very important scavenger for various reactive oxygen species including free radicals²⁾ and singlet oxygen³⁾ produced by oxidative stress *in vivo*. Antioxidative function of bilirubin is significantly effective especially in physiologically relevant concentration of oxygen (2%) *in vivo*.²⁾ Therefore, we expect an antioxidative activity in phycocyanobilin and a possible application of this activity for food preservation or protection of the living cell from oxidative stress. The objectives of this paper are to evaluate the antioxidative activity of phycocyanobilin prepared from *S. platensis* and to compare its activity with several known phytochemicals of which antioxidative activities have recently attracted interest.

Lyophilized crude preparation of phycocyanin extracted from *S. platensis* was kindly supplied from Dainippon Ink & Chemical Inc., Chiba, Japan. The crude extract was brought to 50% saturation in ammonium sulfate and then was centrifuged (1,000 × *g*) for 20 min at 5°C. The precipitate was dissolved in water and dialyzed against 1,000 ml of 0.05 M phosphate buffer (pH 7.8) at 5°C overnight, then applied to a column containing DEAE cellulose (Whatman DE52) equilibrated with the same buffer. The column was washed with a concentration gradient solution of phosphate buffer (0.05 M–0.2 M) at pH 7.8. Blue fractions were collected as partially purified phycocyanin. Phycocyanobilin was prepared from this phycocyanin according to the method described by Carra.⁴⁾ Antioxidative activities of phycocyanobilin and other compounds including quercetin, genistein, catechin, caffeic acid, and chlorogenic acid were evaluated in 80 mM phosphate buffer (pH 7.4) containing 10 μM antioxidant, 4.4 mM linoleic acid, 5 mM sodium cholate, and 10 mM 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), which thermally decomposes and produces peroxy radicals. The reaction mixture was oxidized by AAPH, and linoleic acid hydroperoxides formed during incubation at 37°C were periodically ex-

tracted by chloroform/methyl alcohol (1:1) and determined by HPLC using a silica gel column (YMC-pack SIL, 150 mm × φ4.6 mm) with a UV detector (235 nm). The eluent was n-hexane/isopropyl alcohol/acetic acid (97.8:2:0.2). All chemicals were purchased from Nacalai Tesque (Kyoto, Japan) unless otherwise indicated.

Figure 1 shows the formation of linoleic acid hydroperoxides in the reaction mixture initiated by AAPH. In the reaction mixture without phycocyanobilin, the oxidation of linoleic acid resulted in a linear accumulation of hydroperoxides of the acid and no induction period was observed while phycocyanobilin effectively inhibited the peroxidation of linoleic acid. Antioxidative activities of phytochemicals including catechin, quercetin, genistein, caffeic acid, and chlorogenic acid were also evaluated under the same reaction condition (Fig. 1). Among the chemicals examined, quercetin showed the strongest antioxidative activity, followed by caffeic acid, chlorogenic acid, and catechin. Genistein showed no activity under this condition. These flavonoids and phenolic carboxylic acids

Fig. 1. Antioxidative activities of phycocyanobilin and several phytochemicals and change in absorbance of phycocyanobilin at 610 nm.

Linoleic acid with each compound was oxidized by AAPH, and hydroperoxides formed were determined by HPLC. Each value shows the average from two measurements.

●: control, ■: phycocyanobilin (10 μM), □: catechin (10 μM), ▲: quercetin (10 μM), △: caffeic acid (10 μM), ×: chlorogenic acid (10 μM), ○: genistein (10 μM), ◆: absorbance of phycocyanobilin at 610 nm.

have recently attracted the attention of many researchers. Caffeic acid has a marked radical scavenging activity among other hydroxycinnamic acids such as *p*-coumaric acid and ferulic acid.⁵⁾ A typical flavonol quercetin also acts as an effective scavengers for active oxygen species, especially when they attack plasma lipoproteins and biomembranes.⁶⁾ Other phytochemicals except for genistein also exhibit the same levels of antioxidative activities. Our results indicated that phycocyanobilin has the antioxidative activity equal to those of the flavonoids and phenolic carboxylic acids,⁷⁾ especially in the early stage of oxidation. However, after 120 minutes, the rate of hydroperoxide formation in the phycocyanobilin system was $6.9 \times 10^{-8} \text{ MS}^{-1}$, which was almost the same value as that of the control ($5.9 \times 10^{-8} \text{ MS}^{-1}$). Stoichiometric numbers of radicals trapped by phycocyanobilin are possibly less than phytochemicals tested, or the high speed for radical trapping may have led to rapid exhaustion of active sites of phycocyanobilin.

Antioxidative activities of phycocyanin against several radical species were recently noted.⁸⁾ However, the active sites or the antioxidative mechanisms including whether phycocyanobilin moiety is involved in the activities or not are unknown. Some amino acid residues in a polypeptide chain are known to exhibit antioxidant activities. Wayner *et al.*,⁹⁾ for example, suggested that human serum albumin highly contributes to antioxidation in the plasma, and sulfhydryl groups are responsible for the activity. They also suggested that tryptophan, tyrosine, and histidine residues may trap peroxy radicals in a less polar environment. Since apophycocyanins prepared from various cyanobacteria contain these antioxidant amino acid residues,¹⁰⁾ a possible contribution of the apoprotein to the total antioxidant activity of phycocyanin can not be excluded. A remarkable antioxidative activity of phycocyanobilin obtained in our study, however, suggested that the tetrapyrrol chromophore corresponding to phycocyanobilin is

highly responsible for the activity of phycocyanin. The absorbance of phycocyanobilin (610 nm) rapidly decreased during the induction period, indicating the occurrence of cleavage of conjugated double bonds in the chromophore (Fig. 1, dashed line). Stocker *et al.*²⁾ suggested that antioxidative activity of the free bilirubin in methanol can be ascribed to the reactive hydrogen atom at C-10 which is easily accessible to juxtaposed lipid peroxy radicals formed during lipid oxidation. Phycocyanobilin, however, does not contain the reactive hydrogen at C-10, but contains longer conjugated double bonds than that of bilirubin. The possible cleavage of conjugated double bonds in its tetrapyrrol structure may indicate involvement of a radical addition reaction to the double bonds.

Further studies will be taken to clarify the antioxidative activities of phycocyanobilin under the condition close to the actual living body.

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