Note

Antioxidant Activity of the Phycoerythrobilin Compound Formed from a Dried Korean Purple Laver (*Porphyra* sp.) during *in Vitro* Digestion

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To evaluate the accessibility and function of phycoerythrin (a purple-pigment protein) found in purple laver (*Porphyra* sp.), antioxidant activity of the phycoerythrobilin compound (chromophore of the pigment protein) formed from the dried Korean purple lavers was determined by *in vitro* digestion. Results suggest that the apoprotein of phycoerythrin is readily digested to release the phycoerythrobilin compound during the gastrointestinal digestion process of mammals. The peroxy radical scavenging capacity was 2.7-fold greater in the phycoerythrobilin compound than in the purple laver extracts. The various therapeutic activities of phycoerythrin appear to be associated with the phycoerythrobilin compound released during mammalian gastrointestinal digestion.

Keywords: antioxidant activity, Korean purple laver, *in vitro* digestion, *Porphyra*, phycoerythrin, phycoerythrobilin

Introduction

Various types of edible algae are available as food stuff. Certain purple lavers (*Porphyra* sp.) are the most widely consumed among east Asian countries, especially Japan, where many kinds of purple laver products (e.g., dried, seasoned and toasted) made from Susabi-nori (*Porphyra yezoensis*), Asakusa-nori (*Porphyra tenera*), and Iwa-nori (*Porphyra* sp.) are common. Many Korean purple laver products (dried, and seasoned and toasted) have also recently become popular consumer products in Japan. The Korean purple lavers is very similar to Japanese Iwa-nori. Several sheets of dried purple lavers (nori) are often served with steamed rice and can be consumed worldwide with certain sushi.

Dried purple lavers contain various nutrients (Takenaka *et al.*, 2001) and therapeutic compounds such as Porphyrin (antitumor, antihypertensive, and antihyperlipidemic polysaccharides) (Takahashi *et al.*, 2000), carnosine and anserine (antioxidant dipeptides) (Tamura *et al.*, 1998), and phycoerythrin (pigment protein) (Fujiwara, 1961). Phycoerythrin, one of the phycobiliproteins which are the light-harvesting acces-

sory pigments, is composed of phycoerythrobilin (chromophore, a linear tetrapyrrole compound) and apoprotein (Jiang *et al.*, 1999). Phycoerythrin also has been reported to have hepatoprotective (Soni *et al.*, 2008) and antioxidant (Soni *et al.*, 2009) properties in mammals. In the previous studies, the isolated phycoerythrin has been directly administrated to certain experimental animals to evaluate its therapeutic properties. When phycoerythrin is orally ingested by mammals, the pigment protein is thought to be digested during the gastrointestinal digestion process to release the covalently linked chromophore moiety, the phycoerythrobilin compound. Therefore, the therapeutic activities of phycoerythrin may be due to the formed phycoerythrobilin compound (Fig. 1).

Here, we evaluated the antioxidant activity of the phycoerythrobilin compound formed from the dried Korean purple laver during *in vitro* digestion.

Materials and Methods

Materials Folin-Ciocalteu reagent and 2,2-azobis (2-methylpropionamide)dehydrochloride (AAPH) were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Saffron, pepsin (P7012, from porcine gastric mucosa), and pancreatine (P8096, from porcine pancreas) were pur-

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chased from Sigma (St. Louis, MO, USA). Trolox was obtained from Calbiochem (La Jolla, CA, USA). C-phycoerythrin was obtained from Funakoshi Co., Ltd. (Tokyo, Japan).

All other reagents used were of the highest purity commercially available. The tested samples (1-3) of dried Korean purple lavers were provided from local markets in Seoul, Korea. Japanese purple lavers (Iwa-nori, dried) were purchased from local markets in Kanazawa (samples 4 and 5) and Tottori (sample 6), Japan.

Antioxidant capacity assays The antioxidant's reducing capacity and peroxy radical scavenging capacity were measured by Folin-Ciocalteu assay (FCR) as an electron transfer method and by crocin bleaching assay (CBA) as a hydrogen atom transfer method (Hang *et al.*, 2005). These assays were done in triplicate and expressed as a trolox equivalent.

FCR was done according to the method of Stevanato *et al.* (2004). Color development was performed for 2 h, and absorbance was measured at 760 nm using an ultraviolet/visible Shimadzu spectrophotometer (160A, Kyoto, Japan).

CBA was done by the method of Ordoudi and Tsimidou (2006). Crocin was isolated from saffron (Sigma) by methanol extraction (Lussignoli *et al.*, 1999). The crocin solution was diluted with 0.1 mol/L phosphate buffer (pH 7.0) to obtain 10 $\mu\text{mol/L}$ crocin solution (absorption coefficient of crocin $1.33 \times 10^5 \text{ mol}^{-1} \text{cm}^{-1}$ at 443 nm). After the addition of the sample to 2 mL crocin aqueous solution in a 3-mL capacity cuvette (1-cm length), the reaction mixture was preincubated at 40°C. The peroxy radicals were generated by the addition of 12.5 mmol/L AAPH and the bleaching rate of crocin at 443 nm was monitored at 40°C by the spectrophotometer. After the addition of AAPH, the decrease in absorbance was determined every 3 min for 10 min. Bleaching rates were estimated according to the equation $V_0/V = 1 + K_a[A]/K_c[C]$, where V_0 is the basal bleaching rate of crocin in the absence of antioxidants, V is the bleaching rate of crocin in the presence of antioxidants, $[C]$ and $[A]$ are the concentrations of crocin and antioxidants, respectively. K_a and K_c are the rate constants for the reaction of the peroxy radicals with antioxidants and crocin, respectively. The Trolox equivalent was calculated from the K_a/K_c values.

In vitro digestion *In vitro* digestion was performed based on the method of Laparra *et al.* (2003). Briefly, 30 g dried Korean purple laver 1 and 2 was broken into small pieces with a mortar and pestle. A portion (5.0 g) of each broken laver was added to 90 mL distilled water. The pH was adjusted to 2.0 with 1 mol/L HCl. Freshly prepared pepsin solution (1 g of pepsin in 10 mL of 0.1 mol/L HCl) was added to provide 0.01 g (21,900 units) of pepsin/5 g of laver. After 15 min, pH of the solution was checked and readjusted to pH 2.0, if necessary. The sample was made up to 100 g

Fig. 1. Proposed mechanism of intestinal absorption and therapeutic functions of the purple laver phycoerythrin.

with distilled water and incubated in a shaking water bath (100 strokes/min) at 37°C for 2 h.

Prior to the pancreatin digestion step, the pH of the pepsin digest (pH 2.0) was raised to pH 7.0 by the addition of 1 mol/L NaHCO_3 . Freshly prepared pancreatin solution (0.2 g of pancreatin in 50 mL of 0.1 mol/L NaHCO_3) was added to provide 2.5 mg of pancreatin/5 g of laver. The sample was incubated in a shaking water bath (100 strokes/min) at 37°C for 2 h.

The pancreatin digest was centrifuged at $1,600 \times g$ for 30 min at 4°C to separate soluble and precipitate fractions. The soluble fraction was put on a Sep-pak Vac (5 g) which had been washed with 75% (v/v) ethanol solution followed by distilled water. The column was washed with 50 mL distilled water and then the purple pigment (phycoerythrobilin compound) was eluted with 50 mL of 75% (v/v) ethanol solution.

The eluant was evaporated to dryness, dissolved with a small volume of distilled water, and lyophilized with a freeze dryer (Labconco, Corp., Kansas, MI, USA).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Aliquots (0.1 mL) of Korean purple laver 1 and 2 extracts with or without *in vitro* digestion (after the pancreatic digestion step) were prepared in the sample buffer according to the manufacturer's instructions. A precasted gel (PAGEL, type NPG-520L, ATTO Corp., Tokyo, Japan) was used for electrophoresis on slab gel of 5–20% linear gradient polyacrylamide in the presence of SDS. After electrophoresis on the slab gel, proteins in the gels were stained with coomassie brilliant blue R-250 and destained in acetic acid solution. As standard proteins, a Bio-Rad kit for calibration of molecular weights (Precision plus Protein Standards) was used.

Visible spectra The spectrum was measured with the spectrophotometer at room temperature; quartz cuvettes ($d = 1$ cm) were used. A portion of the laver extract and phycoerythrobilin compound was dissolved in 1 mL distilled water.

Results and Discussion

Purple laver extract The purple pigment protein phycoerythrin was extracted with distilled water from various dried Korean and Japanese purple lavers. Absorbance (565 nm) of Korean and Japanese purple laver extracts (10 g/L) was 1.20 ± 0.05 and 0.45 ± 0.08 (mean $\pm$ SD, $n=3$), respectively, suggesting that the phycoerythrin content in Korean purple lavers is about threefold greater than that in Japanese purple lavers (Fig. 2).

In vitro digestion Gastrointestinal digestion is the most important process in the bioavailability and/or therapeutic properties of the pigment protein phycoerythrin. After oral

administration of the dried purple laver, gastrointestinal proteases appear to digest the pigment protein to release its chromophore moiety (phycoerythrobilin compound). The degree of proteolysis of the pigment protein from the dried purple laver during *in vitro* digestion was therefore evaluated. Although the phycoerythrobilin compound was significantly released from the laver during digestion, the purple color pigment still remained in the precipitate (undigested) fraction. We could not estimate the digestion rate of the phycoerythrobilin compound due to difficulty in assaying the compound in the dried laver. Using the same digestion method, Miyamoto *et al.* (2009) estimated the digestion rate of vitamin B₁₂ in the Korean purple laver to be about 50%.

SDS-PAGE results of the released phycoerythrobilin compound was analyzed (Fig. 3). Although the protein bands with molecular masses of 18–34 kDa (corresponding to phycoerythrin) (Jiang *et al.*, 1999) were found in the purple laver extract (lanes 1 and 2), they completely disappeared following *in vitro* digestion (lanes 3 and 4), strongly suggesting that the apoprotein of phycoerythrin is readily digested to release the phycoerythrobilin compound during the mammalian gastrointestinal digestion process.

Visible absorption spectra The visible absorption spectrum of the Korean purple laver extract (10 g/L) showed absorption maximums at 673.5 ($\lambda_{\max}$: 0.348), 616.0 (0.484), 562.5 (1.144), and 496.0 (0.941) (Fig. 4A), while that of the phycoerythrobilin compound (2 g/L) showed an absorption maximum at 493.5 nm (0.758) (Fig. 4B).

Phycoerythrin can be divided into three main classes

Fig. 2. Color of Japanese and Korean purple laver extracts. Each sample (1 g) of the dried Japanese and Korean purple lavers was powdered and then extracted with 100 mL distilled water for 1 h at 25°C. The extracts were filtrated with filter paper (type 5A) before the experiments. Korean (1–3) and Japanese (4–6) purple laver extracts were put into plastic dishes (30×15 mm). The samples show typical color patterns from the three independent experiments.

Fig. 3. SDS-PAGE of the purple laver extracts with or without *in vitro* digestion. The Korean laver extracts and digests (after pancreatic digestion step) were electrophoresed on slab gel of 5–20% linear gradient polyacrylamide in the presence of SDS. M, molecular mass of standard proteins. The migration patterns of the laver extracts 1 (lane 1) and 2 (lane 2) from Fig. 2, and their digests (lanes 3 and 4, respectively) represent typical patterns for three independent experiments.

depending on their absorption spectrum, B-phycoerythrin (peaks at 545 and 565 nm with a shoulder at 499 nm), R-phycoerythrin (peaks at 499 and 565 nm with a shoulder at 545 nm), and C-phycoerythrin (peak at 565 nm) (Roman *et al.*, 2002). Judging from the absorption peaks of the laver extract (peaks at 496.0 and 562.5 nm with a shoulder at 542.5 nm), the Korean purple lavers used in the experiments appear to contain mainly R-phycoerythrin. The remaining absorption peaks at 673.5 and 616.0 nm may correspond due to allophycocyanin (peak at 650 nm) and phycocyanin (peak at 620 nm) (Eriksen, 2008), respectively.

Antioxidant activity of the purple laver treated with or without *in vitro* digestion To evaluate the antioxidant activity of the Korean purple laver extracts, FCR and CBA were performed to determine the antioxidant's reducing capacity and the peroxy radical scavenging capacity, respectively. As shown in Table 1, the purple laver extracts have about 26% of the antioxidant activity of C-phycocyanin as the peroxy radical scavenger. The peroxy radical scavenging capacity was 2.7-fold greater in the phycoerythrobilin compound (Trolox equivalent, 0.048 ± 0.003 mmol/g) than in the purple laver extracts (0.018 ± 0.001 mmol/g) (Fig. 5).

Soni *et al.* (2009) previously demonstrated the involvement of phycoerythrin in the amelioration of diabetic complications by significantly reducing oxidative stress and oxidized LDL-triggered atherogenesis when purified phycoerythrin was administered to experimental streptozotocin–nicotinamide-induced type 2 diabetic male rats.

C-phycocyanin (a blue pigment protein) found in cyanobacterium *Spirulina plantensis* has been reported to be a

Fig. 4. Visible absorption spectra of the purple laver extract and the prepared phycoerythrobilin compound. The Korean purple laver 1 extract (A) (10 g/L) and phycoerythrobilin compound (B) (2 g/L) were analyzed. The data are typical absorption spectra from the three independent experiments.

Table 1. Antioxidant activity of Korean purple laver extracts.

	Antioxidant activity (Trolox equivalent, mmol/g)	
	FRC	CBA
Korean purple lavers	0.201 ± 0.001	0.018 ± 0.001
C-phycocyanin	0.985 ± 0.001	0.070 ± 0.005

Two grams of the dried Korean purple laver 1, 2 and 3 were powdered and then extracted with 100 mL of distilled water for 1 h at 25°C. Each extract was filtrated with a filter paper (type 5A) and the used in the experiments. C-phycocyanin (2 g/L) was used as the peroxy radical scavenger. An antioxidant's reducing capacity by Folin-Ciocalteu reagent (FCR) and peroxy radical scavenging capacity by crocin bleaching assay (CBA) were determined. Data represent mean $\pm$ SEM from three independent experiments.

potent peroxy radical scavenger *in vitro* and *in vivo* (Bhat and Madyastha, 2000). The chromophore phycocyanobilin of phycocyanin is reduced in mammals to phycocyanorubin (Terry *et al.*, 1993), which can inhibit formation of superoxide radicals by NADPH oxidase complex (McCarty, 2007; Riss *et al.*, 2007). These findings imply that phycoerythrobilin as well as phycocyanobilin can be metabolized to function in mammalian cells. Taken together with the results of the present study, various therapeutic activities of phycoerythrin appear to be associated with the release of phycoerythrobilin during gastrointestinal digestion in mammals.

Fig. 5. Antioxidant activity of the prepared phycoerythrobilin compound. The lyophilized phycoerythrobilin compound (1–5 g/L) was dissolved in small amounts of distilled water and used for FCR and CBA. The values represent means $\pm$ SEM from three independent experiments.

References

- Bhat, V.B. and Madyastha, K.M. (2000). C-phycoerythrin: a potent peroxyl radical scavenger *in vivo* and *in vitro*. *Biochem. Biophys. Res. Comm.*, **275**, 20-25.
- Eriksen, N.T. (2008). Production of phycocyanin-a pigment with applications in biology, biotechnology, foods and medicine. *Appl. Microbiol. Biotechnol.*, **80**, 1-14.
- Fujiwara, T. (1961). Studies on chromoproteins in Japanese nori, *Porphyra tenera*. V. on the sugar components of phycoerythrin. *J. Biochem.*, **49**, 361-367.
- Hang, D., Ou, B., and Prior, R.L. (2005). The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.*, **53**, 1841-1856.
- Jiang, T., Zhang, J. P. and Liang, D. C. (1999). Structure and function of chromophores in R-phycoerythrin at 1.9Å resolution. *Proteins*, **34**, 224-231.
- Laparra, J.M., Vlez, D., Montoro, R., Barber, R., and Farr, R. (2003). Estimation of arsenic bioaccessibility in edible seaweed by an *in vitro* digestion method. *J. Agric. Food Chem.*, **51**, 6080-6085.
- Lussignori, S., Fraccaroli, M., Andrioli, G., Brocco, G., and Bellavite, P. (1999). A microplate-based colorimetric assay of the total peroxyl radical trapping capability of human plasma. *Anal. Biochem.*, **269**, 38-44.
- McCarty, M.F. (2007). Clinical potential of *Spirulina* as a source of phycocyanobilin. *J. Med. Food*, **10**, 566-570.
- Miyamoto, E., Yabuta, Y., Kwak, C.S., Enomoto, T., and Watanabe, F. (2009). Characterization of vitamin B₁₂ compounds from Korean purple laver (*Porphyra* sp.) products. *J. Agric. Food Chem.*, **57**, 2793-2796.
- Ordoudi, S.A. and Tsimidou, M.Z. (2006). Crocin bleaching assay (CBA) in structure-radical scavenging activity studies of selected phenolic compounds. *J. Agric. Food Chem.*, **54**, 9347-9356.
- Riss, J., Decorde, K., Sutra, T., Delage, M., Baccou, J.C., Youy, N., Brune, J.P., Oreal, H., Cristol, J.P., and Rouanet, J.M. (2007). Phycobiliprotein C-phycoerythrin from *Spirulina platensis* is powerfully responsible for reducing oxidative stress and NADPH oxidase expression induced by an atherogenic diet in hamsters. *J. Agric. Food Chem.*, **55**, 7962-7967.
- Roman, R.B., Alvarez-Pez, J.M., Fernandez, F.G.A. and Grima, E.M. (2002). Recovery of pure B-phycoerythrin from the microalga *Porphyridium cruentum*. *J. Biotechnol.*, **93**, 73-85.
- Soni, B., Visavadiya, N.P., and Madamwar, D. (2008). Ameliorative action of cyanobacterial phycoerythrin on CCl₄-induced toxicity in rats. *Toxicol.*, **248**, 59-65.
- Soni, B., Visavadiya, N.P., and Madamwar, D. (2009). Attenuation of diabetic complications by C-phycoerythrin in rats: antioxidant activity of C-phycoerythrin including copper-induced lipoprotein and serum oxidation. *Brit. J. Nutr.*, **102**, 102-109.
- Stevanato, R., Fabris, S., and Momo, F. (2004). New enzymatic method for the determination of total phenolic content in tea and wine. *J. Agric. Food Chem.*, **52**, 6287-6293.
- Takahashi, K., Hirano, Y., Araki, S., and Hattori, M. (2000). Emulsifying ability of *Porphyran* prepared from dried nori, *Porphyra yezoensis*, a red alga. *J. Agric. Food Chem.*, **48**, 2721-2725.
- Takenaka, S., Sugiyama, S., Ebara, S., Miyamoto, E., Abe, K., Tamura, Y., Watanabe, F., Tsuyama, S., and Nakano, Y. (2001). Feeding dried purple laver (nori) to vitamin B₁₂-deficient rats significantly improves vitamin B₁₂ status. *Brit. J. Nutr.*, **85**, 699-703.
- Tamura, Y., Takenaka, S., Sugiyama, S., and Nakayama, R. (1998). Occurrence of anserine as an antioxidative dipeptide in a red alga, *Porphyra yezoensis*. *Biosci. Biotechnol. Biochem.*, **62**, 561-563.
- Terry, M.J., Maines, M.D., and Lagarias, J.C. (1993). Inactivation of phytochrome- and phycobiliprotein-chromophore precursors by rat liver biliverdin reductase. *J. Biol. Chem.*, **268**, 26099-26106.