Antioxidative Activity of the Anthocyanin Pigments Cyanidin 3-*O*-β-D-Glucoside and Cyanidin

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The antioxidative activity of the anthocyanin pigments cyanidin 3-*O*-β-D-glucoside (C3G) and cyanidin (Cy) was examined by using linoleic acid autoxidation, liposome, rabbit erythrocyte membrane, and rat liver microsomal systems. C3G and Cy had antioxidative activity in all systems. Cy had a stronger activity than C3G and the same activity as α-tocopherol (Toc) in the liposome and rabbit erythrocyte membrane systems. In the rat liver microsomal system, Cy and C3G exhibited stronger activity than Toc. These data suggest that the pigments may play an important role in the prevention of lipid peroxidation of cell membranes induced by active oxygen radicals in living systems as dietary antioxidants after ingestion.

Keywords: *Antioxidant; Phaseolus vulgaris L.; anthocyanin; pigments; cyanidin*

In living systems, oxygen species such as hydroxy radicals, superoxide anion radicals, and singlet oxygens are thought to be agents that attack polyunsaturated fatty acids in cell membranes and give rise to lipid peroxidation. Lipid peroxidation is strongly associated with aging and carcinogenesis (Yagi, 1987; Harman, 1982; Cutlar, 1984). Living systems are protected from active oxygen species by enzymes such as superoxide dismutase, glutathione peroxidase, and catalase; they also receive nonenzymatic protection by endogenous antioxidants such as α-tocopherol (Toc), ascorbic acid, β-carotene, and uric acid (Ames et al., 1981).

Recently, it was reported that one of these natural antioxidants contained in dietary plants may play an important role in the prevention of carcinogenesis and in extending the life span of animals and that dietary antioxidants may offer effective protection from peroxidative damage in living systems (Cutlar, 1984; Osawa et al., 1990). Therefore, much attention has been focused on natural antioxidants, and some antioxidants isolated from natural sources with high activity have been reported (Katsuzaki et al., 1993; Okamura et al., 1993; Osawa et al., 1992; Nishina et al., 1991).

Since no attention had been directed to edible pulses as natural sources, we reported screening for antioxidative activity of edible pulses (Tsuda et al., 1993a) and examined the antioxidative activity of pea bean (*Phaseolus vulgaris* L.) extract (Tsuda et al., 1993b). Further, we have found that cyanidin 3-*O*-β-D-glucoside (C3G), an anthocyanin pigment isolated from red bean (*P. vulgaris* L.), showed strong antioxidative activity (Tsuda et al., 1994). C3G is found widely in the human diet of many seeds, fruits, and vegetables, indicating that we ingest many anthocyanin pigments such as C3G through plant-based daily diets. C3G may play an important role as a dietary antioxidant after ingestion in the prevention of lipid peroxidation of cell membranes induced by active oxygen radicals in living systems. Therefore, the antioxidative activity of C3G *in vitro* models such as liposome, rabbit erythrocyte membrane ghost, and rat liver microsome systems was examined. In addition, since C3G may have the potential to produce cyanidin (Cy) by hydrolysis with β-glucosidase of intestinal bacteria after ingestion (Tamura et al., 1980), the comparative antioxidative effect of C3G and its aglycon, Cy, was also studied.

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MATERIALS AND METHODS

Chemicals. C3G was purified from red bean seed coat (*P. vulgaris* L. cv. Honkintoki) by using preparative HPLC. The structure and purity were confirmed by ^{1}H and ^{13}C NMR, FAB-MS, UV–vis, and IR spectra (Tsuda et al., 1994). Cyanidin chloride (Cy) was purchased from Extrasynthese, France. Linoleic acid, egg lecithin, and Toc were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Linoleic Acid System. Autoxidation of linoleic acid in the water–alcohol system was carried out by using the method of Osawa and Namiki (1981). Each sample was added to a solution mixture of linoleic acid (0.13 mL) and 99.0% distilled ethanol (10 mL), with a 50 mM phosphate buffer (pH 7.0, 10 mL); the total volume was adjusted to 25 mL with distilled water. The solution was mixed in a conical flask and incubated at 40 °C. At intervals during incubation, the degree of oxidation was measured by the thiocyanate method (Mitsuda et al., 1966) by reading the absorbance at 500 nm after coloring with $FeCl_2$ and thiocyanate.

Liposome System. Egg lecithin (100 mg) was sonicated in an sonicator with a 10 mM phosphate buffer (pH 7.4). The resulting multilamellar vesicles (MLV) were sonicated in a cup-horn-type sonicator (Insonator 201M, Kubota, Japan) at 120 W for 20 min, by which process small unilamellar vesicles (SUV) were obtained. The SUV solution (10 mg of liposome/mL), 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH), or $CuSO_4$, with a phosphate buffer (pH 7.4), and antioxidants were mixed to produce a final concentration of 1 mg of liposome/mL, 2 mM AAPH, or 1 μM $CuSO_4$ and 1 mM phosphate buffer (pH 7.4). Samples (C3G, Cy) and standard

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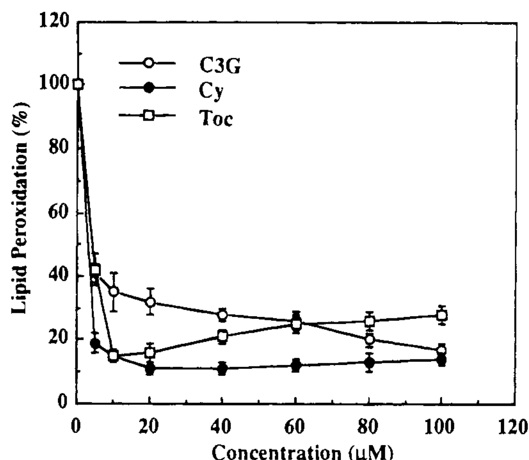

Figure 1. Antioxidative activity of cyanidin 3-*O*-β-D-glucoside (C3G), cyanidin (Cy), and α-tocopherol (Toc) in the linoleic acid system as measured by the thiocyanate method. Reported values are the mean ± SD ($n = 3$). A control containing no added samples on its value represents 100% lipid peroxidation.

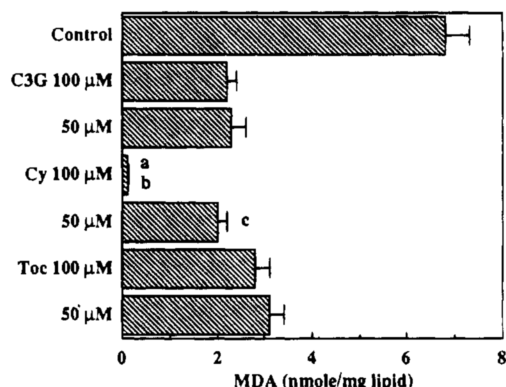

Figure 2. Antioxidative activity of cyanidin 3-*O*-β-D-glucoside (C3G), cyanidin (Cy), and α-tocopherol (Toc) in the liposome system. Lipid peroxidation was induced by 2 mM AAPH. Reported values are the mean ± SD ($n = 3$). (a) $P < 0.001$, compared with 100 μM C3G. (b) $P < 0.05$, compared with 100 μM Toc. (c) $P < 0.05$, compared with 50 μM Toc.

antioxidant (Toc) were dissolved in methanol and added to the reaction mixture. The final concentrations of antioxidants were 50 and 100 μM. The reaction mixture was stored at 37 °C for 6 h, and the formation of malondialdehyde (MDA) was measured by using the HPLC urea method reported by Osawa and Shibamoto (1992). In this method, MDA was reacted with urea and the product, 2-hydroxypyrimidine, was analyzed by HPLC. It is specific to formation of MDA.

Rabbit Erythrocyte Membrane Ghost System. Commercially available rabbit blood (100 mL) was obtained from Japan Biotest Institute Co., Ltd., and diluted with 100 mL of isotonic buffer solution (10 mM phosphate/152 mM KCl, pH 7.4). After centrifugation (1500*g*, 20 min), the blood was washed three times with 100 mL of the isotonic buffer solution and lysed in 10 mM phosphate buffer (pH 7.4). Erythrocyte membrane ghosts were pelleted by centrifugation (20000*g*, 40 min), and the precipitate was diluted to give a suspension (1.0 mg of protein/mL) (Osawa et al., 1987). Peroxidation of the erythrocyte membrane ghosts induced by *tert*-butyl hydroperoxide was carried out according to the method of Ames et al. (1981). Antioxidants were dissolved in methanol, and the final concentrations of antioxidants were 50 and 100 μM. After incubation at 37 °C for 30 min, the formation of MDA was measured by using the HPLC urea method described above.

Rat Liver Microsomal System. Wister rats (8 weeks, 180–200 g) were sacrificed and their livers removed and homogenized. Microsomes were prepared by differential centrifugation according to the method of Slater and Sawyer (1971). Fresh solutions, in 50 mM Tris-HCl buffer (pH 7.4), were prepared each time at the concentration of 2 mg/mL microsomal protein. Samples were dissolved in methanol and added to the microsomal incubation, and final concentrations were 50 and 100 μM. Next, 8 mM ADP, 0.4 mM $FeCl_3$, 0.4 mM EDTA, and 0.4 mM NADPH in 50 mM Tris-HCl buffer (pH 7.4) were added (final concentrations were 2.0, 0.1, 0.1, and 0.1 mM, respectively) and incubated at 37 °C for 30 min according to the method of Osawa et al. (1987), slightly modified. After incubation, the formation of MDA was measured by using the HPLC urea method described above.

Statistics. Statistical analysis was performed by using Student's *t*-test.

RESULTS AND DISCUSSION

Antioxidative Activity in the Linoleic Acid System. Antioxidative activities of C3G, Cy, and Toc as measured by the thiocyanate method in the linoleic acid system are shown in Figure 1. C3G did not show strong antioxidative activity at 5 μM; however, the higher the concentration was, the stronger C3G had antioxidative activity. C3G showed stronger activity than Toc at 100 μM. On the other hand, Cy had a marked inhibition of lipid peroxidation even if the concentration was 5 μM. The extent of activity was stronger than C3G and Toc when the concentration was 5 μM. From these results, it is obvious that C3G and Cy are effective antioxidants and that Cy is stronger antioxidant than C3G and Toc in the autoxidation system when using linoleic acid.

Antioxidative Activity in the Liposome System. A major target of free radical damage is the cellular membrane which contains abundant unsaturated lipids (Aust and Sringen, 1982); rising lipid peroxidation is strongly associated with aging and carcinogenesis. Liposomes have been used extensively as cellular models for in vitro lipid peroxidation studies. Therefore, we used the liposome system for evaluating antioxidants as simple in vitro cellular models. The antioxidative assay in the liposome system usually used is the thiobarbituric acid (TBA) method (Buege and Aust, 1978). However, this assay is not specific to MDA and is interfered with in the presence of anthocyanin pigments because the measured wavelength of the TBA reactive substance is similar to the absorption (λ_{max}) of the pigments. Therefore, we used the HPLC urea method for the determination of MDA level.

Figure 2 shows the antioxidative activity of C3G, Cy, and Toc in the liposome system where lipid peroxidation is induced by AAPH. As shown, C3G and Cy show an inhibitory effect against the formation of MDA. The extent of activity of C3G was as strong as the standard antioxidant, Toc, at 50 and 100 μM. Cy had marked antioxidative activity, and it was more effective than Toc when the concentration was both 50 and 100 μM (significantly different, $P < 0.05$).

Figure 3 shows antioxidative activity of C3G, Cy, and Toc in the liposome system with lipid peroxidation induced by $CuSO_4$. As well as in the case of AAPH, C3G and Cy had strong inhibition of MDA formation. Cy was more effective than Toc and C3G when the concentration was 100 μM. At 50 μM, Cy did not have a stronger antioxidative activity than Toc.

Cy is the aglycon of C3G and is more hydrophobic, indicating that Cy has higher affinity with the liposome and shows stronger antioxidative activity than C3G. In the results, C3G and Cy had antioxidative activity not only in the autoxidation of the linoleic acid system but also in the cell membrane model system such as liposome.

Antioxidative Activity in the Rabbit Erythrocyte Membrane System. Like many other biological

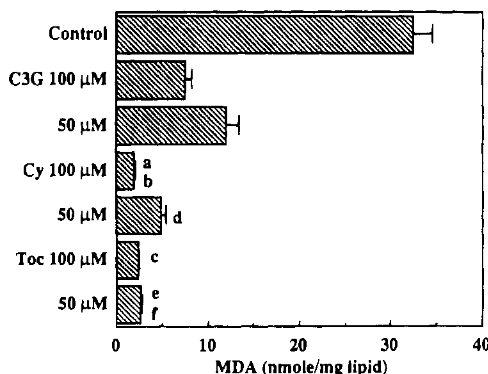

Figure 3. Antioxidative activity of cyanidin 3-*O*-β-D-glucoside (C3G), cyanidin (Cy), and α-tocopherol (Toc) in the liposome system. Lipid peroxidation was induced by 1 μM $CuSO_4$. Reported values are the mean ± SD (n = 3). (a) $P < 0.05$, compared with 100 μM C3G. (b) $P < 0.05$, compared with 100 μM Toc. (c) $P < 0.05$, compared with 100 μM C3G. (d) $P < 0.05$, compared with 50 μM C3G. (e) $P < 0.05$, compared with 50 μM Cy. (f) $P < 0.05$, compared with 50 μM C3G.

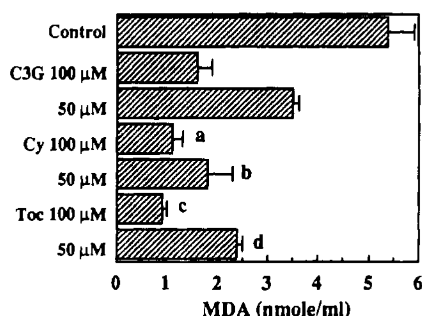

Figure 4. Antioxidative activity of cyanidin 3-*O*-β-D-glucoside (C3G), cyanidin (Cy), and α-tocopherol (Toc) in the rabbit erythrocyte membrane ghost system. Antioxidants of 50 and 100 μM were used for this experiment; details are given under Materials and Methods. Reported values are the mean ± SD (n = 3). (a) $P < 0.001$, compared with 100 μM C3G. (b) $P < 0.01$, compared with 50 μM C3G. (c) $P < 0.05$, compared with 100 μM C3G. (d) $P < 0.001$, compared with 50 μM C3G.

membranes, red blood cell membranes are prone to lipid peroxidation because of their high polyunsaturated lipid content. Therefore, the evaluation of antioxidative activity of C3G and Cy was performed in this system. The results as measured by the HPLC urea method are shown in Figure 4. C3G and Cy showed strong antioxidative activity when the concentration was 100 μM. The activity of C3G was weaker than that of Toc ($P < 0.05$) and Cy ($P < 0.001$). The extent of the activity of Cy was the same as Toc (not significant) and stronger than C3G ($P < 0.001$) at both 100 and 50 μM. C3G did not have stronger activity than Toc ($P < 0.05$). When the concentration was 50 μM, Cy exhibited strong activity. Like the result in the liposome system, the higher affinity of Cy with erythrocyte membrane caused more effective inhibition of MDA formation than C3G.

Antioxidative Activity in the Rat Liver Microsomal System. Microsomes isolated from liver have been shown to catalyze an NADPH-dependent peroxidation of ferric ions and metal chelators, such as ADP (Hochstein and Ernster, 1963). Microsome membranes are particularly susceptible to lipid peroxidation owing to the presence of high concentrations of polyunsaturated fatty acids. We used rat liver microsome as an enzymatic lipid peroxidation system in the presence of ADP, EDTA, and ferric ion and examined comparative antioxidative activity of C3G, Cy, and Toc. Figure 5 shows the antioxidative activity of C3G, Cy, and Toc in the

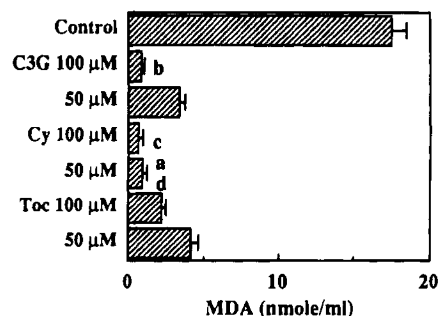

Figure 5. Inhibition of enzymatic lipid peroxidation in the rat liver microsome system by cyanidin 3-*O*-β-D-glucoside (C3G), cyanidin (Cy), and α-tocopherol (Toc). The final concentration of antioxidants were 50 and 100 μM. Details are given under Materials and Methods. Reported values are the mean ± SD (n = 3). (a) $P < 0.001$, compared with 50 μM C3G. (b) $P < 0.01$, compared with 100 μM Toc. (c) $P < 0.01$, compared with 100 μM Toc. (d) $P < 0.001$, compared with 50 μM Toc.

rat liver microsome system as measured by the HPLC urea method. C3G and Cy also had markedly strong antioxidative activity at both the 50 and 100 μM levels in this system. Cy and C3G exhibited stronger activity than Toc when the concentration was 100 μM ($P < 0.01$). Cy had stronger antioxidative activity than C3G and Toc even if the concentration was 50 μM. From these results, we see that Cy and C3G also have strong antioxidative activity in the NADPH-dependent enzymatic lipid peroxidation system.

CONCLUSION

Kakegawa et al. (1987) reported that the synthesis of anthocyanin [cyanidin 3-(6″-malonyl)glucoside] was induced by illumination with UV light in cultures of *Centaurea cyanus* cells. Takahashi et al. (1991) also reported that anthocyanin pigment [cyanidin 3-(6″-malonyl)glucoside] accumulated cells were more resistant to irradiation with UV light, which reduced formation of UV induced pyrimidine dimers. These results indicate that anthocyanin pigments play a role in protecting plant cells against UV-induced damage and also suggest that the pigments may function as antioxidants to protect oxidative damages in animal cells.

C3G is widely distributed in the human diet through seeds, fruits, and vegetables, indicating that we ingest many anthocyanin pigments from plant-based daily diets. The results we obtained in in vitro lipid peroxidation systems suggest that the pigments may play a role in prevention of lipid peroxidation of cell membranes induced by active oxygen radicals in living systems as dietary antioxidants after ingestion. Cy may be produced from cyanidin glucosides by hydrolysis with β-glucosidase of intestinal bacteria after ingestion, indicating that Cy may act as antioxidant rather than C3G in living systems and that C3G can be classified as a proantioxidant. However, since these experiments have been carried out *in vitro*, it is necessary that the investigation for antioxidativeactivity of the anthocyanin pigments be done in an *in vivo* system. We will carry out a feeding experiment with these anthocyanin pigments by using an *in vivo* system.

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