

Protective Effects of Boysenberry Anthocyanins on Oxidative Stress in Diabetic Rats

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The protective effects of major boysenberry anthocyanins (BoAnt) against oxidative stress were investigated in streptozotocin (STZ)-induced diabetic rats. Increases in the concentration of plasma thiobarbituric acid reactive substances (TBARS), and in the liver 8-hydroxy deoxyguanosine (8-OH dG)/deoxyguanosine (dG) ratio and also in the liver GSSG/GSH ratio, which were all observed in STZ-injected rats, were restored or tended to be restored to the level of the control rats when a diet with BoAnt was given to the diabetic animals. The susceptibility of the liver homogenate of the diabetic rats to the oxidation by AAPH was relieved when BoAnt was fed to them. These results suggested that BoAnt was effective in protecting the development of *in vivo* oxidation involved with diabetes.

Keywords: boysenberry, anthocyanins, diabetes, oxidative stress, thiobarbituric acid reactive substances (TBARS), 8-hydroxy deoxyguanosine (8-OH dG)

Physiological functions of anthocyanins contained in some of the reddish-purple colored peels of fruits and vegetables have been investigated for their protective effects against certain diseases. It has been demonstrated that anthocyanins have the following physiological functions: atherogenic index-lowering effects of rubrobrassicin (cyanidin 3-sophoroside-5-monoglucoside) (Igarashi *et al.*, 1990), serum cholesterol-lowering effect of nasunin from peel of eggplant (Kayamori & Igarashi, 1994), triacylglycerol-lowering effect of malvin (Igarashi & Inagaki, 1991), protective effects of dietary cyanidin 3-O-glucoside on liver ischemia-reperfusion injury (Tsuda *et al.*, 1999b), promoting effect of anthocyanogen for regeneration of rhodopsin (Tronche *et al.*, 1967), protective effect of cyanidin on oxidation of low density lipoprotein (Satue-Gracia *et al.*, 1997), and preventive effects of nasunin and acylated anthocyanins from red cabbage on paraquat-induced oxidative stress in rats (Kimura *et al.*, 1999; Igarashi *et al.*, 2000). Anthocyanins are expected to have even more physiological functions which differ from those above.

Since it is generally pointed out that diabetes induces *in vivo* peroxidation, anthocyanins and other compounds showing anti-oxidative activity *in vitro* are expected to prevent the progress of peroxidation involved with diabetes.

In this paper, we examined whether boysenberry anthocyanins, which are widely cultivated in New Zealand and used as raw materials of jams and beverages and as food colorants, are effective in protecting rats, in which diabetes has been induced by streptozotocin (STZ) injection, from oxidative stress. Our investigations on diabetic rats sought to identify the effects of dietary boysenberry anthocyanins on the amounts of plasma and urine glucose, on the concentrations of plasma and liver thiobar-

bituric acid reactive substances (TBARS), on the formation of 8-hydroxy deoxyguanosine (8-OH dG) as a DNA oxidation product and, further, on the liver glutathione concentrations. The effects of feeding of the BoAnt on susceptibility of the liver homogenate of these rats to the oxidation was also investigated. The suppressive effect of grape anthocyanin dye on increase in the amounts of urine and serum glucose in diabetic rats was reported; this is thought to have different anthocyanins from those of boysenberry among its constituents (Jankowski *et al.*, 2000). However, the effects of boysenberry major anthocyanins which are composed of three kinds of anthocyanins on the plasma and liver oxidative status and on the liver and kidney 8-OH dG concentrations, as well as the amounts of urine and serum glucose, have not yet been reported. Absorbability of boysenberry anthocyanins from the gastrointestinal tract was also determined to deduce its antioxidative activity *in vivo*.

Materials and Methods

Preparation of anthocyanins Boysenberry juice concentrate, which was produced from ripe fresh or frozen boysenberries by milling, pressing, filtering and evaporating them to 65% brix, was the gift of the Berryfruit Export NZ, Ltd. (Nelson, New Zealand).

The concentrate was passed through a column of Amberlite XAD-7 (Organo Ltd., Tokyo) after its dilution with 3% acetic acid. After washing the column with 0.1% acetic acid, the adsorbed anthocyanins were eluted with 30% and 50% ethyl alcohol, successively. The eluate with 30% ethyl alcohol, which was evaporated and dissolved with small amounts of 0.01% HCl-MeOH, was added to diethylether to precipitate the anthocyanins. The precipitate was then dried *in vacuo* and used for animal experiments as boysenberry major anthocyanins (BoAnt).

Anthocyanins in the BoAnt (peaks a, b and c) in Fig. 2 (A) were identified as cyanidin 3-O-glucosylrutinoside, cyanidin 3-

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O-digluconide and cyanidin 3-O-glucoside, respectively, by ^1H -, ^{13}C - and 2D-NMR, and FAB-MS spectrometries of those compounds, which were isolated from BoAnt by Sephadex LH-20 column chromatography using $\text{MeOH-AcOH-H}_2\text{O}=10\text{-}1\text{-}9$ (v/v) as developing solvent. These three anthocyanins were reported earlier to be contained in boysenberry (Wightman & Wrolstad, 1996). The purity of BoAnt as anthocyanin was about 95% when calculated using the isolated a, b and c as a standard for HPLC.

Animals and diets Male Wistar strain rats, 6 weeks-old and each weighing about 95 g, were purchased from Shizuoka Laboratory Animal Center (Hamamatsu) and fed a commercial diet (F-2, Funahashi Farms, Funahashi) for 3-days before being used in the experiment. The rats were kept under controlled conditions with a 12-h light and 12-h dark cycle (6:00–18:00 light), a temperature range of 22–24°C, and a relative humidity of 40–60%. Except those in the control group, the animals were injected with 60 mg of STZ (50 mg/ml of 0.9% NaCl solution) per kg of body weight after fasting overnight, and their urine collected for 24 h. Rats that excreted a significant amount of glucose in their urine, in comparison with those without STZ injection, were used as diabetic rats for the experiment 48 h after the injection. Diabetic rats were divided into two groups of 6 rats each; one group was given a basal diet (STZ group) and the other was given a basal diet with 0.1% BoAnt (STZ+BoAnt group). Six rats injected with 0.9% NaCl solution without Stz were used as a control group (Con group) and given a basal diet. Each diet was given for 28 days, and an equal amount was given to each animal. Water was also given *ad libitum*. The composition of the diets is shown in Table 1. During days 24 to 26 of the experiment, urine was collected for measurement of glucose.

At all times, the rats were cared for according to the institutional guidelines of Yamagata University.

Collection and preparation of blood and organs Blood was collected by cardiac puncture from rats that had been anesthetized with Nembutal (50 mg/ml, Dainippon Pharmaceutical Co., Osaka) after 10 h of food deprivation (from 03:00–06:00 to 13:00–16:00) at the end of the 28-day feeding period. A 0.1 ml aliquot of the blood was added to 1.9 ml of physiological saline with gentle shaking, and the mixture was centrifuged at $1000\times g$

for 10 min to obtain plasma for measuring the plasma TBARS concentration. Centrifuging the blood at $1000\times g$ for 15 min separated the serum for glucose measurement. The liver and kidneys were detached after blood collection, and a section of each of them was frozen until needed to measure the concentration of lipid, 8-OH dG or glutathiones.

Lipid analysis The lipid from a frozen liver was extracted and purified by the method of Folch *et al.* (1957). The lipid content in the extract was determined by weighing it.

Measurement of TBARS The plasma TBARS concentration was determined by the method of Yagi (1976) and is expressed as nmol malondialdehyde per ml of blood. The liver TBARS concentration was measured by the method of Uchiyama and Mihara (1978) using the homogenate, which was prepared by homogenizing the liver (right lobe) with 0.1 M of ice-cold phosphate buffer (pH 7.4) containing 1 mM EDTA, according to the method of Del Boccio *et al.* (1990). For the measurement of kidney TBARS, 0.5 g of a frozen kidney was homogenized with 9 fold of cold 1.15% KCl and the method of Uchiyama and Mihara (1978) was followed.

Measurement of reduced- and oxidized-form glutathiones A frozen liver was homogenized with 10 volumes of 0.4 N perchloric acid containing 2.0 mM EDTA. The supernatant, which was obtained by centrifuging the homogenate at $10,000\times g$ for 5 min under 5°C, was filtered with a 0.2 μm filter. Reduced- and oxidized-form glutathiones (GSH and GSSG, respectively) in the filtrate were determined by an ion-pairing reverse phase HPLC coupled to a coulometric detector according to the method of Harvey *et al.* (1989).

Measurement of 8-OH dG A section from the liver or kidney (0.2 g) was homogenized with 3 ml of cold 0.15 M NaCl/0.1 M EDTA (pH 8.0) solution in a homogenizer with a Teflon pestle on ice. A 1.5 ml aliquot of the homogenate was centrifuged at $1500\times g$ for 15 min at 4°C. DNA from the obtained precipitate was extracted and purified using a DNA extractor (WB kit, sodium iodide method) purchased from Wako Pure Chemical Ind., Ltd. (Tokyo). The final nucleic acid pellet was suspended with 100 μl of cold 1 mM EDTA (pH 8.0), digested to deoxynucleotides by treatment with nuclease P1 and alkaline phosphatase (Kasai *et al.*, 1987), and then centrifuged at $10,000\times g$ for 3 min at 4°C. The amounts of nucleic acid and 8-OH dG in the supernatant were analyzed by HPLC coupled with a UV detector and a coulometric detector, using a column of Ultrasphere-ODS (4.6 \times 250 mm, Beckman Co., Ltd.) and mobile phase of 10 mM potassium phosphate containing 8% MeOH. The flow rate was

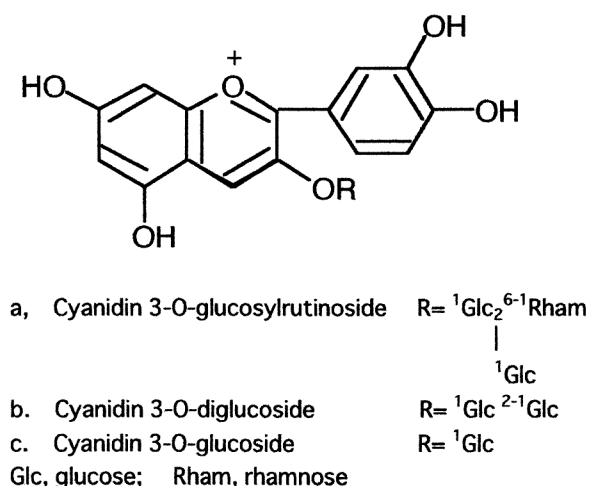


Fig. 1. Chemical structures of boysenberry major anthocyanins.

Table 1. Composition of the diets (%).

Ingredient	Con diet	STZ diet	STZ+BoAnt diet
Casein	20.0	20.0	20.0
α -Cornstarch : sucrose = 2 : 1	65.5	65.5	65.4
Cellulose powder	5.0	5.0	5.0
Corn oil	5.0	5.0	5.0
Mineral mixture ¹	3.5	3.5	3.5
Vitamin mixture ²	1.0	1.0	1.0
Boysenberry anthocyanin (Ant)	—	—	0.1

Con diet, basal diet fed to the rats of control group; STZ diet, basal diet fed to the streptozotocin-injected rats; STZ+BoAnt diet, basal diet with BoAnt fed to the streptozotocin-injected rats. ¹AIN-93-VX and ²AIN-93G-MX (Reeves *et al.*, 1993), which contains 25 g of choline bitartrate per 1 kg, were obtained from Oriental Yeast Co., Tokyo.

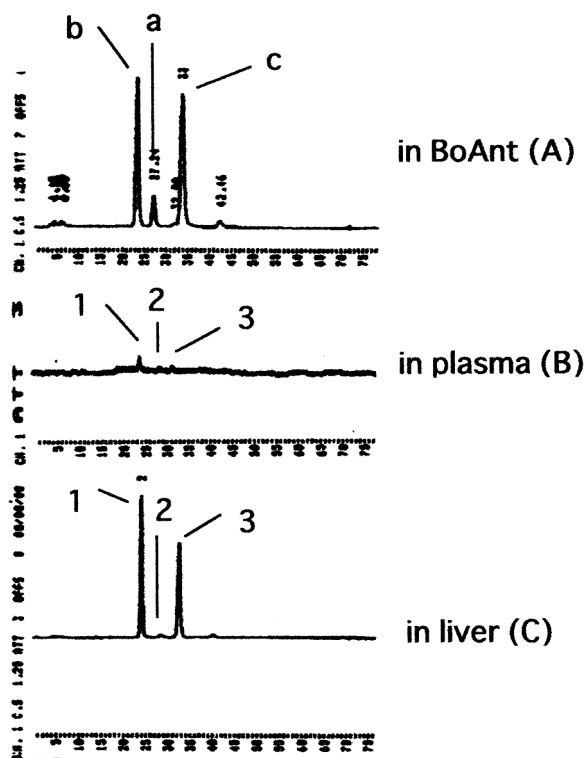


Fig. 2. HPLC chromatograms of boysenberry major anthocyanins (BoAnt) (A) and anthocyanins contained in the plasma (B) and liver (C). Boysenberry major anthocyanins (BoAnt) eluted from a Diaion column with 30% MeOH, were analyzed in an ODS column (Develosil C-30-UG-5, 4.6×250 mm; Nomura Chemical Co., Seto-shi, Aichi), using a linear gradient of 0–100% of solvent B (88% HCOOH-AcOH-H₂O-CH₃CN=3-3-20-4, v/v) in solvent A (88% HCOOH-AcOH-H₂O=1-1-9, v/v) over the course of 180 min at a flow rate of 0.8 ml/min. Detection at 534 nm. (A) BoAnt: a, cyanidin 3-O-glucosylrutinoside; b, cyanidin 3-O-diglucoside; c, cyanidin 3-O-glucoside. Peaks 1, 2 and 3 in both the plasma (B) and liver (C) were identified as cyanidin 3-O-diglucoside, cyanidin 3-O-glucosylrutinoside and cyanidin 3-O-glucoside by coinjection with the respective anthocyanins isolated from BoAnt.

settled at 0.3 ml/min, and nucleic acid was detected at 260 nm. The potential set for the dual coulometric detector was 150 and 300 mv for electrodes 1 and 2. The amount of 8-OH dG in DNA was expressed as the ratio of the amount of 8-OH dG to deoxyguanosine (dG).

Measurement of plasma and liver anthocyanins Wistar strain rats weighing about 150 g were administered 30 mg of BoAnt in 0.5 ml of H₂O 30 min before the collection of blood by heart puncture and subsequent detachment of liver under anesthesia with Nembutal (Dainippon Pharmaceutical Co.). The plasma (0.5 ml), which was obtained by centrifuging the blood at 2000×g for 10 min at 5°C, was immediately added with 1.5 ml of 0.1 ml of trifluoroacetic acid, followed by application to the top of a Diaion column (1×4 cm). Anthocyanins adsorbed on the column were eluted with 30, and 50% MeOH after washing the column with 0.1% AcOH. The combined eluate was evaporated and dissolved with a very small amount of solvent A (88% HCOOH-AcOH-H₂O=1-1-9, v/v) for HPLC analysis. The analytical condition is described in the legend to Fig. 2.

For determination of liver anthocyanins, 0.6 g of the detached liver was homogenized with 2 ml of 5% trifluoroacetic acid, followed by centrifugation at 3000×g for 20 min at 4°C. The supernatant (0.2 ml) was mixed with 4 ml of 0.5% acetic acid, and

then was applied to the top of a Diaion column (1×4 cm). Anthocyanins adsorbed on the column were treated in the same way as those used for preparing anthocyanin fractions from the plasma and analyzed by HPLC.

Oxidative susceptibility of liver homogenate The effect of ingested BoAnt on the susceptibility of the liver homogenate to the oxidation was determined by measuring TBARS formed by oxidation of the liver homogenate with the 2,2'-azobis (amino-propane) hydrochloride (AAPH).

The liver homogenate of each rat was prepared as described for the liver TBARS measurement. A reaction mixture composed of 3 ml of 10 mM AAPH solution in a 40 mM phosphate buffer (pH 7.4) and 0.5 ml of the liver homogenate was incubated in light-shaded test tubes at 37°C. Aliquots were withdrawn and placed in the test tube which contained 10 μl of 0.03 M butylhydroxytoluene in MeOH, at 0, 2 and 4 h after the reaction for the TBARS measurement, which was done by the method of Uchiyama and Mihara (1978).

Protein concentration was determined following Lowry *et al.* (1951), using bovine serum albumin as the standard.

Statistical analyses Values are given as means of standard errors. The homogeneity of the variance between treatments was verified by Bartlett's test. Differences among groups were determined by Duncan's multiple range test after a one way analysis of variance (ANOVA) (Duncan, 1957). Significant differences in the means were inspected at $p < 0.05$.

Results

Food intake, body weight gain, organ weights and glucose concentration The results are shown in Table 2. Although food intake did not differ among Con, STZ and STZ+BoAnt groups, body weight gain at 28 days showed a significantly lower value in the STZ group compared to that of the Con group; this was also true of the STZ+BoAnt group. However, these lower values in the STZ group tended to be relieved in the STZ+BoAnt group, although not to a statistically significant level. The amounts of excreted urine and serum glucose concentration showed significant increases in the STZ group compared with the Con group, indicating that the former group of animals suffered from diabetes. The increase in the amount of excreted urine glucose and serum glucose concentration in the STZ declined to a suppressed state in the STZ+BoAnt group, but not to a significant level. An increase in the kidney weight (% of body weight) in the STZ group, which was compared to that of the Con group, also tended to be suppressed slightly in the STZ+BoAnt group.

TBARS, 8-OH dG and glutathione concentrations Changes in the TBARS, reduced- and oxidized-form glutathione concentrations, and the ratio of 8-OH dG to dG as biochemical markers concerned with diabetes are also shown in Table 2. The increase in plasma TBARS concentration in the STZ group, compared to that of the Con group, tended to be suppressed in the STZ+BoAnt group. Increases in both the liver and kidney 8-OH dG/dG ratio in the STZ group, compared with those of the Con group, were suppressed and also tended to be suppressed slightly in the STZ+BoAnt group.

Absorption of BoAnt HPLC chromatograms of anthocyanin fractions separated from plasma and liver of rats orally administered BoAnt are shown in Fig. 2 (B) and (C). Peaks 1, 2 and 3 in both plasma and liver were identified as cyanidin 3-O-

Table 2. Effects of boysenberry anthocyanins on the body weight gain, organ weight, liver lipid and oxidative status in diabetic rats.

Group	Con	STZ	STZ+BoAnt
Initial body weight (g) ¹	103±1	98.0±3.0	97.7±3.2
Food intake (g)	391±1	391±1	392±1
Body weight gain (g/28 days)	126±3 ^a	50.3±16.4 ^b	72.8±10 ^b
Liver weight (% of body weight)	3.51±0.12 ^b	4.17±0.09 ^a	4.00±0.17 ^a
Liver total lipid (mg/ g of liver)	39±2	35±4	38±2
Kidney weight (% of body weight)	0.72±0.02 ^b	1.1±0.1 ^a	0.90±0.07 ^{ab}
Glucose			
in urine (mg/urine collected for 48 h)	0.294±0.014 ^b	4429±1142 ^a	2895±686 ^a
in serum (mg/ml)	3.15±0.13 ^b	5.33±0.45 ^a	4.28±0.44 ^{ab}
TBARS			
in plasma (nmol/ml of blood)	5.04±0.69 ^b	9.90±1.86 ^a	5.97±0.28 ^{ab}
in liver (nmol/g of liver)	131±7	131±19	131±9
in kidney (nmol/g of kidney)	211±16	246±23	186±13
8-OH dG {(8-OH dG(pmol)/dG(pmol))×10 ⁶ }			
in liver	0.142±0.010 ^{ab}	0.244±0.045 ^a	0.127±0.015 ^b
in kidney	0.117±0.010 ^b	0.179±0.019 ^a	0.161±0.042 ^a
Liver GSH (mmol/kg of liver)	3.74±0.20 ^b	3.95±0.15 ^{ab}	4.46±0.10 ^a
Liver GSSG (mmol/g of liver)	0.049±0.012 ^b	0.108±0.020 ^a	0.059±0.012 ^b
Liver GSSG/GSH ratio (%)	1.31±0.30 ^b	2.80±0.58 ^a	1.34±0.28 ^b

Values are means±SE of 5–6 rats per group. Values with a different superscript letter are significantly different at $p<0.05$.

Con, group fed the Con diet; STZ, group fed the STZ diet; STZ+BoAnt, group fed the STZ+BoAnt diet.

¹Initial body weight of the Con group is the body weight at 48 h after the injection of physiological saline, and the initial body weight of the STZ and STZ+BoAnt groups are the body weight at 48 h after the injection of streptozotocin.

diglucoside, cyanidin 3-O-glucosylrutinoside and cyanidin 3-O-glucoside, respectively by co-injection with the respective anthocyanins isolated from BoAnt, but the peaks in the plasma were very low.

Susceptibility to lipid peroxidation As shown in Fig. 3, the TBARS value of the liver homogenate in the STZ group, in which the homogenate was oxidized by AAPH, showed higher value than that of the Con group after 2 h of incubation, while the the TBARS value of the STZ+BoAnt group was lower than that of the STZ group after the same period.

Discussion

The serum glucose concentration, the amount of excreted urine glucose and the weight ratio of kidneys to body weight are known to be increased, and the body weight gain to be decreased in rats in which diabetes is induced by the injection of Stz (Ree *et al.*, 1995). Since the kidney weight/body weight ratio, the serum glucose concentration and the amount of urine glucose, all of which were more increased in the STZ group than in the Con group, tended to be suppressed slightly in the STZ+BoAnt group, BoAnt may be effective in protecting against the development of diabetes.

Increases in the plasma TBARS, the liver and kidney 8-OH dG/dG ratio, and liver GSSG in the STZ group indicated that diabetes promoted the lipid peroxidation of the blood, damage to DNA and easy oxidation of *in vivo* antioxidants as reported (Miyake *et al.*, 1998; Ree *et al.*, 1995). Moreover, an increase in the 8-OH dG in the liver and kidney indicated that both are target organs that undergo oxidative damage by diabetes. Lower value in liver 8-OH dG in the STZ+BoAnt group, compared with that in the STZ group, suggests that BoAnt is effective in protecting against *in vivo* oxidation. Since all of the cyanidin 3-O-glucosylrutinoside, cyanidin 3-O-diglucoside and cyanidin 3-O-glucoside as components of BoAnt showed DPPH (1,1-diphenyl-2-picrylhydrazyl) radical and superoxide anion radical scavenging activities by photometric and ESR measurements (data not shown), respectively, antioxidative activities of these anthocyanins are

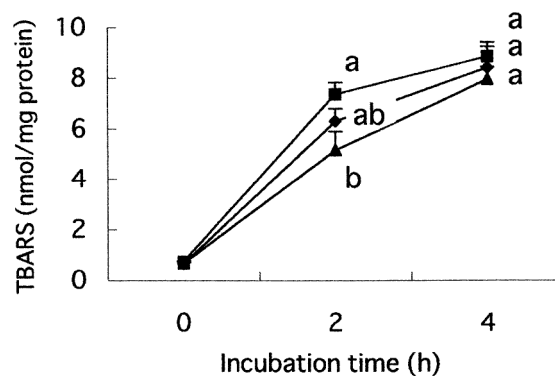


Fig. 3. Effect of dietary boysenberry anthocyanins on the susceptibility of liver homogenate to lipid peroxidation induced by AAPH. Values not sharing a common letter are significantly different at $p<0.05$ within each indicated time. ◆, Con group; ■, STZ group; ▲, STZ+BoAnt group.

believed to play an important role in suppressing changes in these biomarkers by diabetes. Each anthocyanin in the BoAnt is thought to act instead of GSH to save it from consumption, because BoAnt also has antioxidative activity.

An increase in susceptibility of the liver homogenate of diabetic rats (STZ group) to the oxidation by AAPH may suggest that the concentration of the liver antioxidants α -tocopherol and ascorbic acid decreased in these rats more than in control rats. A greater decrease in the susceptibility of the liver homogenate to oxidation in the STZ+BoAnt group than in the STZ group may suggest that BoAnt and/or the liver antioxidants which were held at a higher level by the presence of BoAnt, acted antioxidatively against oxidation of liver homogenate by the AAPH. However, it may also be necessary to determine lipid compositions of the liver homogenates in each group, because differences in the lipid composition may affect susceptibility of the liver homogenate to oxidation.

The identification of cyanidin 3-O-diglucoside, cyanidin 3-O-glucosylrutinoside, and cyanidin 3-O-glucoside in the liver of

rats administered BoAnt and a report describing the absorption of cyanidin 3-O-glucoside from the gastrointestinal tract (Tsuda *et al.*, 1999a; Miyazawa *et al.*, 1999) may support that at least a portion of BoAnt absorbed from the gastrointestinal tract acted antioxidatively. However, as the intestinal contents were a strong purple color, indicating the absorbability of BoAnt may be very low, and as the total content of cyanidin 3-O-diglucoside, cyanidin 3-O-glucosylrutinoside and cyanidin 3-O-glucoside in liver was very little (30–40 µg/liver), it is necessary to determine precisely if the absorbed anthocyanins and/or their metabolite acted antioxidatively to suppress increase in TBARS in the liver homogenate by AAPH. Since the purity of BoAnt as an anthocyanin was about 95%, it may also be necessary to determine whether the other minor components in BoAnt are partly concerned with the decrease in susceptibility of liver homogenate to oxidation, and also with relief of the oxidative stress in diabetic rats.

As the BoAnt was composed of cyanidin 3-O-glucosylrutinoside, cyanidin 3-O-diglucoside and cyanidin 3-O-glucoside, it is very interesting to investigate in future which of these components is the most effective in relieving the oxidative stress.

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