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Enhanced Absorption of Anthocyanins after Oral Administration of Phytic Acid in Rats and Humans

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Many studies on the bioavailability of polyphenols have been reported. However, the relative urinary excretions of AC are also low, ranging from 0.004% to 0.1%. By contrast, other polyphenols show higher urinary excretion levels. Here, we studied the enhancing effects of phytic acid (IP_6) on absorption of blackcurrant anthocyanins (BCAs) in rats and humans. In rats after oral administration of BCAs (as 241 mg of AC/kg body weight) in IP_6 (0%, 0.25%, 0.5%, 1%, 2.5%) solution, the ACs recovery in urine was increased dependent on IP_6 dose. These results suggest that the IP_6 enhances gastrointestinal absorption of ACs. At the further analysis of IP_6 enhancement effect in rat, whereas BCAs were normally passed through the stomach and duodenum within 2 h, in IP_6 group, after 2−6 h post-administration, stomach and jejunum content's weights were specifically heavy, and large amounts of ACs were also detected in stomach, duodenum, and jejunum. These results suggested that the mixture of BCAs and IP_6 reduced the gastrointestinal motility. Prolongation of ACs residue in gastrointestinal tract then caused the enhancing effects of IP_6 on absorption of AC. In the human study, each subject was orally administrated a BCA beverage containing BCA concentrate (AC 4 mg/kg body weight), 1% of IP_6, and 1% of sodium citrate as a pH stabilizer. Both the plasma level and the urinary excretion of AC were increased as compared to BCA administration without IP_6. AC intake with IP_6 may increase the bioavailability of AC to the comparative level as other polyphenols. Yet, phytic acid, being a strong chelator of important minerals, contributes to mineral deficiencies. An interference with iron uptake has been reported. Safety tests are therefore necessary before high dose IP_6 can be used in foods.

KEYWORDS: Anthocyanin; absorption; blackcurrant; phytic acid; bioavailability

INTRODUCTION

The anthocyanins (ACs) are a group of naturally occurring phenolic compounds responsible for the color of many flowers, fruits (particularly berries), and vegetables. Dietary ACs have attracted considerable interest because of their health benefits that include a reduction in the risk of coronary heart disease and prevention of several chronic diseases (*1*). Blackcurrant (*Ribes nigrum* L.) berries and juice are rich in ACs and are consumed throughout the world. We reported previously on the development of a powdered concentrate of blackcurrant ACs (BCAs) from a commercial source (*2*). BCAs consist of four ACs: delphinidin-3-rutinoside (D3R), delphinidin-3-glucoside (D3G), cyanidin-3-rutinoside (C3R), and cyanidin-3-glucoside (C3G). The molecular structures of these compounds are summarized in **Figure 1A**. In previous studies, we demonstrated that oral intake of BCAs has several beneficial effects, including antioxidant effects (*3*), improved vision (*4*), and improved blood circulation (*5*). As a primary component of BCAs, D3R also has unique physiological effects that include relaxation of ciliary smooth muscles (*6*) and reduction of peripheral vascular resistance (*7*).

Many researchers have investigated the kinetics and extent of polyphenol absorption by measuring plasma concentrations and/or urinary excretion in adults after the ingestion of a single dose of a polyphenol (*8*). These studies showed ACs are very rapidly absorbed and eliminated, but that they are relatively poorly absorbed as compared to other polyphenols such as daizin (42.3%), catechin (18.5%), and hesperidin (8.6%) (*8*). It is possible these values may have been underestimated as some important metabolites may have been ignored or the methods used may need to be optimized for the analysis of AC. In our

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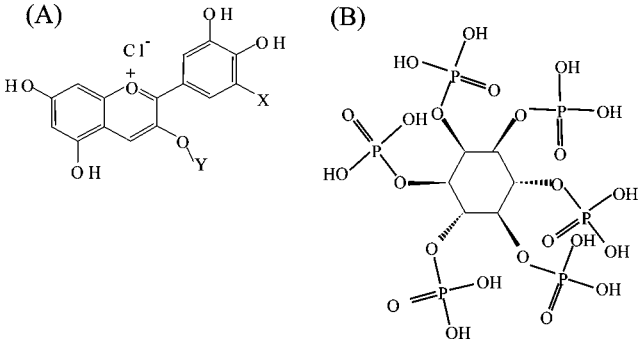

Figure 1. (**A**) Structure of the four anthocyanins in blackcurrants. Delphinidin-3-rutinoside (D3R; X = OH, Y = glucose-rhamnose), delphinidin-3-glucoside (D3G; X = OH, Y = glucose), cyanidin-3-rutinoside (C3R; X = H, Y = glucose-rhamnose), and cyanidin-3-glucoside (C3G; X = H, Y = glucose). (**B**) Structure of phytic acid (IP_6).

recent study, we calculated the pharmacokinetic bioavailability of either orally or intravenously administered ACs in rats by measuring the plasma concentration of D3R (*9*). Relative to an intravenous injection, oral administration of D3R resulted in real bioavailability (0.49 ± 0.06%). We detected only small amounts of the metabolite 4′-*O*-methyl-D3R in the plasma and were unable to detect either anthocyanidin (aglycone) or glucuro- or sulfoconjugates in these samples (*9*). Although C3G (*10*) and pelargonidin-3-*O*-*β*-glucoside (*11*) have been reported to form glucuronyl and sulfate conjugates or a methylated form after oral administration, we have demonstrated previously in humans and rats that D3R and C3R are absorbed in the gastrointestinal tract and are detected mainly in blood and urine as unmetabolized (intact) forms (*12*). In human studies, plasma concentrations of ACs are reported to be very low (10−50 nmol/L), with relative urinary excretion also being low at approximately 0.1% of intake (*12*). These results suggest that ACs are absorbed with poor efficiency as compared to other polyphenols.

However, in a recent study on rats, we observed that bile duct cannulation in a Bollman-type cage significantly increased the bioavailability of orally administered D3R (*9*). The bioavailability of D3R was increased 18.14 ± 6.24%, indicating that absorption was approximately 40 times greater than that in free moving animals without cannulation. We hypothesized that this effect may act by minimizing immobilization stress and reducing gastrointestinal motility. These results indicate that to increase the bioavailability of AC, it is necessary to prevent motility of gastrointestinal motility without having other adverse effects on function. Harada reported that the combination of acylated AC prepared from the purple sweet potato tuber and citric acid slightly enhanced the absorption of ACs (unpublished data). While looking for the combination of food ingredients and AC that increased the bioavailability of AC, we found a mixture of AC and phytic acid was effective.

Phytic acid (*myo*-inositol hexaphosphate, IP_6) is considered historically to be an antinutrient. Structurally, IP_6 contains six phosphates, as shown in **Figure 1B**, and has a strong chelating action, binding minerals such as calcium, iron, and zinc and causing a decrease in their bioavailability in human and animal models. However, a recent study reported that phytic acid has a wide range of beneficial effects including antioxidant, anticarcinogenic, hypoglycemic, and hypolipidemic properties (*13*). IP_6 is considered to be an antioxidant agent as it is a potent inhibitor of iron-catalyzed hydroxyl radical formation by chelating free iron and then blocking its coordination site (*14*). Epidemiological studies have shown a lower incidence of colon cancer in populations consuming a vegetarian-type diet, although the mechanism of this action remains unclear. Furthermore, lower inositol phosphates, such as IP_4 and IP_3, may play roles in mediating cellular responses and have been observed to have a function in secondary-messenger transduction systems. Under Japanese food sanitation laws, there are no limitations on the use of IP_6 as an acidulant. As it is well-known that ACs are stable in aqueous solutions with a high pH, IP_6 is used as an effective stabilizer due to its acidity (*15*).

In the present study, we studied the enhancing effects of IP_6 on the absorption of AC from a BCA concentrate in rats and humans and examined the mechanism of this effect.

MATERIALS AND METHODS

Chemicals. HPLC standards of D3R, D3G, C3R, and C3G were obtained as flavylium chloride salts prepared by recrystallization as described previously (*2*). BCA concentrate was prepared from commercial blackcurrant juice by the methods described in this earlier report (*2*). The concentrate had an AC content of 24.1% and consisted of D3R (10.92%), C3R (8.99%), D3G (3.08%), and C3G (1.11%). The purity was confirmed by HPLC UV−vis spectrometry. Elderberry (*Sambucus nigra*) AC concentrate powder was purchased from Iprona A. G. (Lana, Italy) and had an AC content of approximately 20.4%, measured by 520 nm UV−vis spectrometry using C3G as a tentative standard. Bilberry (*Vaccinium myrtillus*) AC concentrate powder was also purchased from Tokiwa Phytochemicals Co. Ltd. (Chiba, Japan). The AC content of this powder was approximately 28.4%, measured by 520 nm UV−vis spectrometry using C3G as a tentative standard. A 50% solution of IP_6 (food additive grade) was purchased from Tsuno Food Industrial Co., Ltd. (Wakayama, Japan). All other nutrients, reagents, and chemicals used in the study were purchased from commercial sources.

Rat Dose-Dependency Study. Thirty-six male Wistar rats, aged 7 weeks and 140−160 g body weight, were obtained from Clea Japan Co., Ltd. (Tokyo, Japan). The rats were housed individually in stainless-steel wire-mesh cages at 23 ± 1 °C with a 12-h light−dark cycle. The animals were given free access to tap water and commercial diet (MF, Oriental Yeast Co. Ltd., Tokyo, Japan). All of the rats were handled in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (*16*). After the feeding period, food was withheld for 16 h. A 1 g amount of BCA concentrate was dissolved in 20 mL of water containing either IP_6 (0%, 0.25%, 0.5%, 1.0%, 2.5%) or 5% citric acid. The rats were assigned randomly to six groups, and the BCA concentrates (1 g/kg body weight, AC 241 mg/kg body weight) were then administered orally to the animals in each designated group by direct stomach intubation. Urine samples were collected 0−4, 4−8, and 8−24 h after BCA administration.

Comparative Study of IP_6 and Phytin in Rats. Fifteen male Wistar rats, aged 9 weeks and 230−250 g body weight, were obtained from Clea Japan Co., Ltd. (Tokyo, Japan), and reared under conditions identical to those described above. After the feeding period, food was withheld for 16 h. A 415 mg amount of BCA concentrate (equivalent to 100 mg of AC) was dissolved in 20 mL of water, containing 1.0% IP_6 solution or 1.336% phytin solution (Ca salt of IP_6, at the same concentration as the 1.0% IP_6 solution). The rats were assigned randomly to three groups, followed by the oral administration of BCA concentrates (415 mg/kg body weight equivalent to 100 mg of AC/kg body weight) to the rats in each designated group by direct stomach intubation. A 24-h urine sample was collected from each rat following administration.

Analysis of IP_6 Enhanced Effect in Rats. Twenty-four male Wistar rats, aged 8 weeks and 180−190 g body weight, were obtained from Clea Japan Co., Ltd. (Tokyo, Japan), and reared under the conditions described above. After the feeding period, food was withheld for 16 h. BCA concentrate (415 mg equivalent to 100 mg of AC) was dissolved in 20 mL of water or 2.5% IP_6 solution. The rats were assigned randomly to two groups, and AC concentrate (AC 100 mg/kg body weight) was administered orally to the animals in each designated group by direct stomach intubation. Before and after 2, 4, and 6 h post-

administration, three rats in each of the groups were killed under diethyl ether anesthesia by withdrawing blood from the inferior vena cava using a heparinized needle and syringe. Blood and the contents of the stomach, duodenum, jejunum, and ileum were collected from each rat.

Confirmation of IP_6 Enhancing Effect with Other Berry ACs in Rats. Twenty male Wistar rats, 9 weeks of age and 230−250 g body weight, were obtained from Clea Japan Co., Ltd. (Tokyo, Japan), and reared under the conditions described above. After the feeding period, food was withheld for 16 h. A 490 mg aliquot of elderberry AC concentrate powder (AC 20.4%) or 352 mg of bilberry AC concentrate powder (AC 28.4%) was dissolved in 20 mL of water or 1.0% IP_6 solution. The rats were assigned randomly to four groups, and AC concentrate (AC 100 mg/kg body weight) was administered orally to the animals in each designated group by direct stomach intubation. A 24 h urine sample was collected from each rat following administration.

Human Study. Six healthy male subjects (73.5 ± 16.9 kg body weight, 38.2 ± 3.4 years of age) were enrolled in this randomized, crossover study. The study was performed according to the Helsinki Declaration and was approved by the local Ethical Committee. The day before the experiment, the subjects did not consume any type of food rich in anthocyanins, such as vegetables, fruit, or juice. They also did not ingest any food or beverages with the exception of water in the 12-h period prior to the experiment. The BCA-IP_6 beverage was prepared by dissolving 4.15 g/L BCA concentrate in 1% IP_6 and 1% sodium citrate solution. Sodium citrate was used as a pH-adjusting agent (pH 3.17) to make the solution easier to drink. On the day of the experiment, the subjects drank either the BCA-IP_6 beverage that contained 16.6 mg of BCA concentrate/kg body weight (equivalent to 4 mg of AC/kg body weight) or a control beverage containing only the BCA concentrate at the same concentration as the test beverage (i.e., 4 mg of AC/kg body weight). Blood samples (10 mL) were collected at baseline and 1, 2, 4, 6, and 8 h after administration. During the experiment, the subjects drank 200 mL of water every 2 h until 8 h after administration, with urine samples being collected prior to administration, and then at 2-hourly intervals (0−2, 2−4, 4−6, 6−8) until 8 h, and then between 8 and 24 h. Three hours after administration of the BCA-IP_6 or control beverage, the subjects were served an anthocyanin-free lunch, consisting of only a rice ball (225 g) with salt. Nine hours after administration of beverage, the subjects were served an anthocyanin-free supper, consisting of 10 pieces of sushi. Each experiment was separated by a wash-out period of 6-days duration.

Sample Preparation. Plasma samples were prepared by a slight modification of the method of Tsuda et al. (*17*). The plasma was obtained immediately from the collected blood by centrifugation at 1600*g* for 15 min at 4 °C. Separation of the plasma was completed within 30 min and then acidified with a 1/40 volume of 6 N HCl. Four milliliter aliquots of human plasma or 1 mL aliquots of rat plasma were applied to Sep-Pak C_{18} cartridges (Waters, Milford, MA), which had been washed with 10 mL of methanol containing 5% TFA and equilibrated with 10 mL of 10 mmol/L oxalic acid prior to use. After being washed with 10 mmol/L oxalic acid, the ACs were eluted with methanol containing 5% TFA, and the eluate then evaporated carefully to dryness in vacuo below 35 °C. The dried residue was redissolved in 200 μL of 3% phosphoric acid, and a 100 μL aliquot of this solution was injected into an HPLC system for AC analysis. This method was validated as described in our recent report (*12*). Urine samples were acidified with a 1/40 volume of 6 N HCl and 10 mL aliquots applied to Sep-Pak plus C_{18} ENV cartridges, conditioned before use in the same manner as that described for the plasma samples. After the cartridge was washed with 10 mmol/L oxalic acid (10 mL), the adsorbed ACs were eluted with methanol (5.0 mL) containing 5% TFA. The eluate was evaporated in vacuo below 35 °C, and the resulting residue was redissolved in 3% phosphoric acid (200 μL). A 100 μL aliquot of this solution was then subjected to HPLC analysis. The method used to measure the AC amount of the organ content samples was a slight modification of the method of He et al. (*18*). Briefly, four solvents (methanol, acetone, methanol water mixture (60:40; v/v), and double distilled water, all acidified with 1% TFA) were studied to determine their extraction efficiency. Samples of each organ content equivalent to 10 times the weight of the extracting solvent were processed. A Polytron homogenizer (Kinematica AG, Switzerland) was used to facilitate this extraction. The resulting suspension was then sonicated for 3 min, followed by centrifugation at 4000 rpm (3000*g*) for 10 min at 4 °C. The supernatants were collected and evaporated in vacuo below 35 °C, with the resultant residue being redissolved in a small volume of acidified water and then applied to a Sep-Pak C_{18} plus cartridge (Waters, Milford, MA). The cartridges were washed with 10 mL of methanol containing 5% TFA and equilibrated with 10 mL of 10 mmol/L oxalic acid prior to use. After being washed with 10 mmol/L oxalic acid, the ACs were eluted with methanol containing 5% TFA, and the eluate was then evaporated carefully to dryness in vacuo below 35 °C. The dried residue was redissolved in 200 μL of 3% phosphoric acid, and a 100 μL aliquot of this solution was injected into an HPLC system for AC analysis.

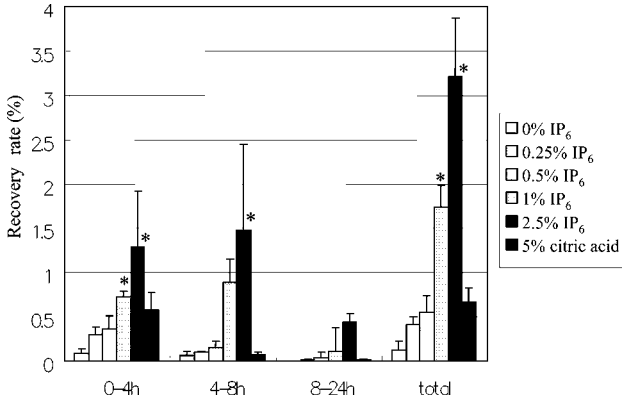

Figure 2. The time-course of changes in recovery rate (%) in urine of BCAs in rats after oral administration of a single dose of BCAs with a range of doses of IP_6 solution (0%, 0.25%, 0.5%, 1%, 2.5%) and 5% citric acid solution. Values are expressed as the mean ± SE for six rats.

Analysis of Anthocyanins. The identification and quantification of the four anthocyanins were performed using a HP 1100 Series HPLC system equipped with a Zorbax SB C-18 column (4.6 mm × 250 mm, particle size 5 mm) and a photodiode array detector at 520 nm (*13*). Injection was performed by means of an autosampler, with a 100 μL fixed loop. Elution was performed using a solvent system consisting of a mixture of solvent A (0.5% phosphoric acid) and solvent B (methanol), applied as a linear gradient from 80% A/20% B (v/v) to 77% A/23% B (v/v) for 15 min, then held at 77% A/23% B (v/v) for a further 8 min at a flow rate of 1.0 mL/min. The eluted constituents were identified by measuring the photodiode array UV−vis spectra from 200 to 600 nm.

The areas of the peaks of D3R, C3R, D3G, and C3G were proportional to the amounts injected within the range 0.2−400 ng. The standard curves were linear over this concentration range, and the detection limit was 0.1 ng in each instance. Standard curves for the four purified ACs were prepared, and the amount and content of the four ACs in each sample were calculated using the respective standard curve. The amount of BCAs was given as the sum of the amount of D3R + C3R + D3G + C3G. Only the approximate quantities of elderberry and bilberry ACs were determined, as we could not obtain highly purified standard products of their ACs to prepare standard curves and evaluate the detection limits. The approximate AC content of elderberry and bilberry was calculated as the sum of the content of all of the ACs measured by 520 nm UV−vis spectrometry, calculated using C3G as the approximate standard.

Statistical Analysis. Statistical analyses were carried out using SPSS version 10.02 for Windows with the data expressed as mean ± S.E.M. For statistical evaluation, the time-course of changes in recovery rate (%) in urine of BCAs in rats (**Figure 2**) was analyzed by one-way ANOVA with the Dunnet post hoc test. Weight and AC content in each organ were analyzed using Student's *t*-test after the confirmation of variance by *F*-test (**Tables 1** and **2**). Other data for the human study were analyzed using the paired *t*-test after the confirmation of variance by *F*-test (**Tables 3** and **4**).

Table 1. Weight of Organ Contents[a]

	stomach (g)		duodenum (mg)		jejunum (mg)		ileum (mg)	
h	2.5% IP_6	0% IP_6	2.5% IP_6	0% IP_6	2.5% IP_6	0% IP_6	2.5% IP_6	0% IP_6
0	0.36 ± 0.10	0.36 ± 0.10	165.9 ± 15.7	165.9 ± 15.7	712.6 ± 91.7	712.6 ± 91.7	1194.2 ± 105.8	1194.2 ± 105.8
2	5.98 ± 0.73*	0.19 ± 0.07	218.3 ± 52.5	190.9 ± 25.3	2488.4 ± 282.0*	1084.2 ± 38.7	2128.8 ± 251.7	1528.5 ± 133.4
4	7.52 ± 0.60*	0.19 ± 0.01	126.3 ± 12.9	185.1 ± 29.8	1834.2 ± 281.6*	680.6 ± 44.2	1829.2 ± 327.3	1042.3 ± 175.6
6	2.92 ± 0.40*	0.22 ± 0.09	153.7 ± 12.0	167.9 ± 28.2	1097.8 ± 113.4*	563.4 ± 78.1	1233.0 ± 221.8	850.4 ± 142.7

[a] Average ± SE, $n = 3$. *Significant difference at $P < 0.05$ versus 0% IP_6.

Table 2. AC Contents in Each Organ (μg)

	plasma[a]		stomach		duodenum		jejunum		ileum	
h	2.5% IP_6	0% IP_6	2.5% IP_6	0% IP_6	2.5% IP_6	0% IP_6	2.5% IP_6	0% IP_6	2.5% IP_6	0% IP_6
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2	1.93 ± 0.30	1.29 ± 0.32	11.45 ± 1.49*	0.29 ± 0.16	125.24 ± 38.68	5.40 ± 4.93	431.92 ± 122.19	495.14 ± 184.11	373.66 ± 119.69*	3338.64 ± 502.32
4	2.15 ± 0.16*	0.09 ± 0.01	4.74 ± 0.82*	0.01 ± 0.00	23.86 ± 5.36*	0.23 ± 0.17	218.43 ± 28.68*	4.45 ± 3.25	642.47 ± 166.54	345.22 ± 282.69
6	1.00 ± 0.08*	0.05 ± 0.02	2.48 ± 0.79	0.02 ± 0.01	5.94 ± 3.79	0.24 ± 0.06	136.23 ± 34.28	2.50 ± 1.39	344.36 ± 26.32*	81.94 ± 41.39

[a] g/mL in plasma (average SE, $n = 3$). *Significant difference at $P < 0.05$ versus 0% IP.

Table 3. Mean AC Concentration in Plasma (ng/mL)[a]

	baseline	1 h	2 h	4 h	6 h	8 h
control	0.000 ± 0.000	0.119 ± 0.047	0.457 ± 0.135	0.373 ± 0.327	0.108 ± 0.014	0.137 ± 0.052
BCA-IP_6	0.391 ± 0.262	5.171 ± 1.741*	2.428 ± 0.988[#]	2.621 ± 0.725**	0.149 ± 0.077	0.253 ± 0.084

[a] Average ± SE, $n = 6$. Significant difference at *, $P < 0.05$; **, $P < 0.01$; and [#], $P < 0.1$ versus control.

Table 4. Mean AC Recovery in Urine (μg)[a]

	baseline	0–2 h	2–4 h	4–6 h	6–8 h	8–24 h	24 h total
control	0.320 ± 0.181	38.20 ± 23.02	24.31 ± 9.65	5.09 ± 2.44	1.90 ± 0.72	9.69 ± 2.54	79.19 ± 31.12
BCA-IP_6	0.352 ± 0.083	105.07 ± 29.04**	89.38 ± 29.02[#]	71.13 ± 30.64[#]	29.97 ± 6.17**	60.02 ± 16.10*	355.57 ± 95.17*

[a] Average ± SE, $n = 6$. Significant difference at *, $P < 0.05$; **, $P < 0.01$; and [#], $P < 0.1$ versus control.

RESULTS

Rat Dose-Dependency Study. Urine samples were collected 0−4, 4−8, and 8−24 h after oral administration of either BCA dissolved in IP_6 solutions (0%, 0.25%, 0.5%, 1.0%, 2.5%) or 5% citric acid. **Figure 2** shows the time-course of changes in the total excretion of the four ACs and the cumulative excretion levels of these ACs in the rats. None of these anthocyanins were detected in urine prior to administration. After oral administration of BCA in IP_6 solutions, the recovery of ACs in urine in the first 24 h increased dependent on the IP_6 dose. At the maximum dose of IP_6, the recovery of AC was significantly 20 times higher than that without IP_6. The enhanced absorption associated with IP_6 was clearly greater than that with the positive control, 5% citric acid. Interestingly, in the groups administered an IP_6 concentration <0.5%, the ACs were excreted rapidly in the urine within 4 h of BCA administration, followed by a decrease in excretion levels. At high IP_6 concentrations >0.5%, the ACs were also rapidly excreted within the first 4 h, although the peak of AC excretion occurred between 4 and 8 h. The cumulative urinary excretion of ACs was >3% of the amount administered orally, a level not greatly different from that of other polyphenols. The intact forms of the four ACs were detected mainly in the urine (data not shown), similar to that seen in our recent studies (*9*, *12*). The urinary volume and pH of each treatment group were not significantly different (data not shown). These results suggest that IP_6 enhances gastrointestinal absorption of AC. To investigate this enhancing effect of IP_6, the AC concentration in plasma and gastrointestinal content were measured after administration of BCAs with or without IP_6, and a comparison study of IP_6 and phytin (Ca salt of IP_6) was carried out in another group of rats.

Comparative Study of IP_6 and Phytin in Rats. We compared the AC absorption enhancing effects of IP_6 and phytin. Phytin is primarily a calcium salt of IP_6 and has a weak chelating effect on minerals. The concentration of phytin was set at 1.336% to remove the amount of calcium as a 1.0% phytin−IP_6 solution. The AC absorption enhancing effect of IP_6 was reconfirmed at different dosages of BCAs (AC 100 mg). In the IP_6 group ($n = 5$), the mean urinary recovery of BCAs (0.87 ± 0.16%) was 5.8 times higher than that in the control group (0.15 ± 0.03%). The AC absorption enhancing effect was not observed in the phytin group (0.21 ± 0.09%). These results suggested that the chelating effect of IP_6 may participate in the AC absorption enhancing effect of the compound.

Analysis of IP_6 Enhancing Effect in Rats. To investigate the AC absorption enhancing effect of IP_6, we measured the AC concentration in plasma and gastrointestinal organ contents (stomach, duodenum, jejunum, and ileum) following administration of BCAs (AC 100 mg) with or without the highest dose of IP_6 (2.5%). The results of organ content weights are summarized in **Table 1**, and organ content's ACs are shown in **Table 2**. The time-course of changes in AC concentrations in rat plasma following administration of BCAs is also summarized in **Table 2**. In both groups, none of the ACs was detected in plasma before administration. In the control group without IP_6, there was an increase in plasma AC concentration to 1.29 ± 0.32

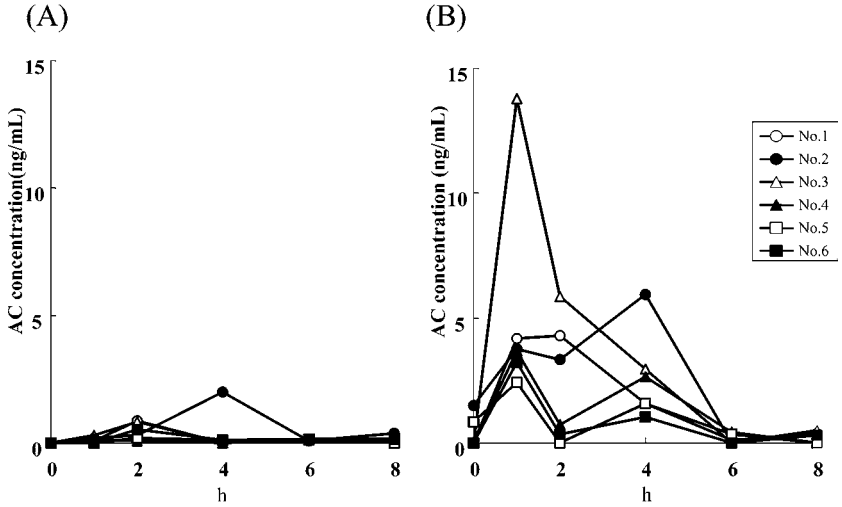

Figure 3. The time-course of changes in BCA plasma concentrations in humans after oral intake of a single dose of control beverage or BCA-IP_6 beverage in six subjects. (**A**) Control beverage group involving intake of BCA concentrate (4 mg of AC/kg body weight). (**B**) BCA-IP_6 beverage group involving intake of BCA concentrate (4 mg of AC/kg body weight) dissolved in 1% IP_6 and 1% of sodium citrate solution (pH 3.17).

μg/mL 2 h following oral administration of BCAs. At 4 and 6 h post-administration, the plasma concentration of ACs decreased to 0.09 ± 0.01 and 0.05 ± 0.02 μg/mL, respectively. These results are similar to those reported in our recent study (*12*). In the group administered both BCA and IP_6, there was an increase in the mean plasma AC concentration to 1.93 ± 0.30 μg/mL 2 h post-administration, which increased further to 2.15 ± 0.16 μg/mL by 4 h. At 6 h post-administration, the mean plasma concentration of ACs had decreased but remained at 1.00 ± 0.08 μg/mL. These results reflected the urinary excretion data and suggested that IP_6 enhances the absorption of AC into the plasma and excretion in urine.

The weight of the contents in the rat stomach following administration of BCAs is summarized in **Table 1**, and the corresponding AC concentrations are shown in **Table 2**. In the control group, the weight of the stomach contents was similar to that before administration, and no ACs were detected in any of the samples. This implied that the BCAs had passed through the stomach within 2 h. However, in the IP_6 group, the weight of the stomach contents was significantly heavier than that in the control group 2−6 h post-administration. ACs amounts were significantly higher than those of the control group in the stomach 2 and 4 h post-administration. These results suggested that the combination of BCAs and IP_6 reduced stomach motility.

In the duodenum, the weight of contents after administration of BCAs was not remarkably different in either group 2−6 h post-administration. However, ACs were detected only in the duodenum during this period in the IP_6 group. This indicates that the BCAs had passed through the duodenum within 2 h in the control group. However, in the IP_6 group, the ACs remained in the duodenum up to 6 h post-administration. At 4 h post-administration, a significant difference was confirmed against the control group.

With regard to the contents of the jejunum, there was no remarkable difference in the control group during the 6 h period following administration. The ACs were detected in the jejunum of these control animals at levels similar to those of the IP_6 group 2 h post-administration only. This finding indicated the BCAs had reached the jejunum within 2 h and had passed through by 4 h. In the IP_6 group, the weight of the jejunum contents was significantly heavier than that in the control group between 2 and 6 h post-administration. ACs were also detected in the jejunum between 2 and 6 h post-administration. At 4 h post-administration, a significant difference was confirmed against the control group.

There was also no remarkable difference in the weight of the ileum's contents 2−6 h after administration of BCAs in either group. However, ACs were detected in the ileum 2 h post-administration only in the control group with the significant difference against IP_6 group. This finding implied that the BCAs had reached the ileum within 2 h in these control animals. By contrast, at 6 h post-administration, ACs in the IP_6 group were significantly higher than those of the control group in the ileum. Also, the bulk of the ACs had not reached the ileum by 2 h, suggesting that the combination of BCAs and IP_6 reduced gastrointestinal motility, especially in the stomach.

Confirmation of the IP_6 Enhancing Effect with Other Berry ACs in Rats. To confirm the absorption enhancing effect of IP_6, we measured the urinary recovery rate of ACs in the 24 h period following administration of ACs derived from other berries. In the control groups of elderberry and bilberry, the urinary recovery rates of ACs 24 h after administration were 0.140 ± 0.03% and 0.280 ± 0.09%, respectively, values that were similar to those measured in the BCA study. The enhancing effect of IP_6 was also effective with both types of berry ACs, with absorption being 4.3 times greater with elderberry ACs (0.600 ± 0.09%) and 10.6 times greater with bilberry ACs (2.96 ± 0.37%).

Human Study. Throughout the study, all of the subjects remained in relatively good health, and no adverse side effects were observed. It was confirmed that the human plasma and urine samples of the two study groups contained a negligible amount of anthocyanins prior to intake of BCA.

Figure 3**A** shows the time-course of changes in the concentrations of ACs in each control subject after ingestion of BCA concentrate alone (16.6 mg/kg body weight providing 4 mg of AC/kg body weight). The plasma AC concentrations in each subject were quite variable but low, being almost less than 1 ng/mL. The maximum concentration of ACs was different in the subjects and occurred at either 1 or 2 h after administration. This result was the same as that reported in our recent study, but different from that for other polyphenols (e.g., the peak for isoflavone occurs between 6 and 8 h). In the BCA-IP_6 group, the dose of ACs in the beverage was the same as in controls (BCA concentrate 16.6 mg/kg body weight providing 4 mg of AC/kg body weight), dissolved in 1% IP_6 and 1% of sodium

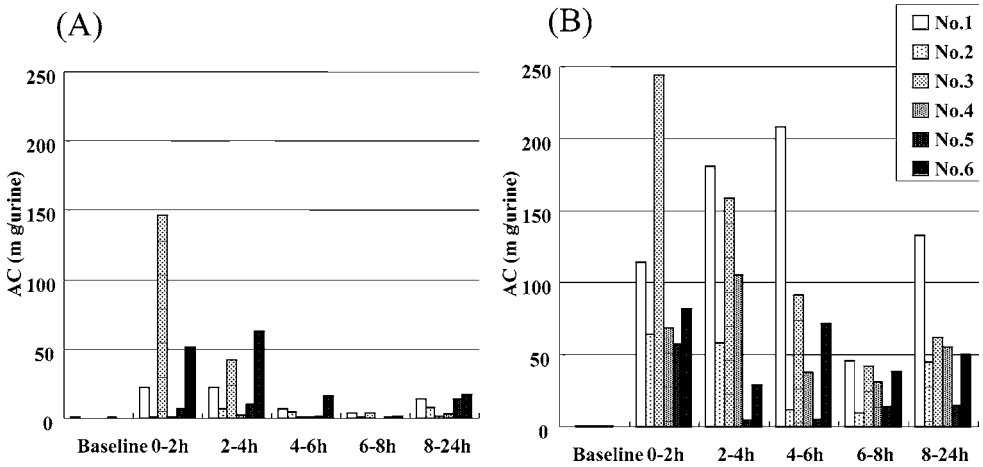

Figure 4. The time-course of changes in the level of urinary excretion of ACs in humans after oral intake of a single dose of control beverage or BCA-IP_6 beverage in six subjects. (**A**) Control beverage group involving intake of BCA concentrate (4 mg of AC/kg body weight). (**B**) BCA-IP_6 beverage group involving intake of BCA concentrate (4 mg of AC/kg body weight) dissolved in 1% IP_6 and 1% of sodium citrate solution (pH 3.17).

citrate solution. **Figure 3B** shows the time-course of changes in the concentrations of ACs in each subject after ingestion of BCA-IP_6 beverage. The plasma AC concentrations of all of the subjects were clearly increased, with the maximum plasma AC concentration 1 or 2 h after administration being different in each subject. The mean plasma AC concentration in both groups is summarized in **Table 3** with the BCA-IP_6 group always having a significantly high concentration at 1 and 4 h after administration. **Figure 4A** shows the time-course of changes in the level of urinary excretion of ACs in the control beverage drinking group (BCA concentrate alone). Although the excretion time of the ACs was variable in each subject, the profile was similar to that observed in our recent study (*12*) (i.e., the excretion peaks occurred within 4 h, and then gradually decreased). **Figure 4B** shows the time-course of changes in the urinary excretion levels of ACs in each subject following ingestion of BCA-IP_6 beverage. Urinary excretion of ACs in all of the subjects was markedly increased. The maximum excretion time of ACs was also different in each subject. For example, in subject NO.3 this occurred in the first 2 h after administration, while in subject NO.1 the peak occurred between 4 and 6 h. The average AC excretion in urine in both groups is summarized in **Table 4**. Comparison of the mean values showed the BCA-IP_6 group always had a higher excretion level during the 8 h following administration. At 0−2, 6−8, and 8−24 h, the cumulative excretion amount had a significant difference between both groups. Moreover, the high level of AC excretion was prolonged until between 0 and 6 h. These results were consistent with the results from our studies on rats. The four ACs (D3R, C3R, D3G, and C3G) were detected mainly in both the plasma and the urine as intact forms (data not shown).

DISCUSSION

Interactions between phytochemicals, or between their secondary products and synthetic pharmaceutical drugs, can potentiate their biological activity in humans, or alternatively interfere with the expected efficacy (*19*). Walton et al. reported that the flavonol, quercetin-3-glucoside, inhibited C3G absorption in vitro (*20*). In contrast, in our study we found that the combination of ACs and IP_6 had positive effects on AC absorption.

Recently, considerable attention has been paid to the biological functions of dietary flavonoids, with ACs, the natural pigments of the flavonoid family, having been shown to have multiple biological effects (*4*, *5*, *21*). In view of these effects, the bioavailability of these compounds is regarded as an important issue. However, in an earlier study (*8*), we found that the urinary excretion of ACs was relatively low, ranging from 0.004% to 0.1%, with these values being quite small as compared to other polyphenols. We calculated the pharmacokinetic bioavailability of BCAs in rats and found that oral administration of D3R resulted in real bioavailability ($0.49 \pm 0.06\%$) as compared to intravenous injections. While this value was greater than that for other ACs, it was markedly smaller than for other polyphenols (*9*). We also observed a rise in bioavailability following oral administration of ACs resulting from immobilization stress associated with reduced gastrointestinal motility.

In the present study, we evaluated the potential of a combination of IP_6 and AC to induce immobilization of gastrointestinal motility in both rats and human, thereby increasing the bioavailability of AC.

In our dose-dependency study in rats, following oral administration of BCA (241 mg of AC/kg body weight) in IP_6 (0%, 0.25%, 0.5%, 1%, 2.5%) solutions, the recovery of ACs in the urine in the first 24 h increased dependent on the IP_6 dose. At the maximum dose of IP_6, the recovery of AC was 20 times higher than that without IP_6. This enhanced absorption caused by IP_6 was obviously greater than that with the positive control, 5% citric acid. Urinary volume and pH in the groups was not remarkably different. Taken together, these results suggest that IP_6 enhanced gastrointestinal absorption of AC. The cumulative urinary excretion of ACs was greater than 3% of the oral administration amount, a level that was not greatly different from other polyphenols. At high IP_6 concentrations greater than 1.0%, although the ACs were excreted rapidly between 0 and 4 h, peak AC excretion was delayed until between 4 and 8 h. It is therefore possible that the mixture of BCAs and IP_6 may remain in the gastrointestinal tract for up to 8 h.

To analyze the enhancing effect of IP_6 on AC absorption in rats, we measured plasma AC levels and the weight of contents in gastrointestinal organs including the stomach, duodenum, jejunum, and ileum. In the IP_6 group, there was a sustained increase in plasma AC concentrations that corresponded to the levels of urinary excretion. At 4 and 6 h after administration, plasma AC concentrations were significant higher than those of the control group. In the control group, the weights of the organ contents were not remarkably different at any time,

whereas ACs were only detected in the jejunum and ileum 2 h after administration of the BCAs. This finding implies that BCAs normally pass through the stomach and duodenum within 2 h. However, in the IP_6 group, the weight of the stomach and jejunum contents were significantly heavier than those in the control group through 2−6 h after administration. A large amount of ACs was also detected in the stomach, duodenum, and jejunum 2−6 h post-administration in the IP_6 group. Only small amounts of ACs were detected in the ileum in the IP_6 group as compared to controls, indicating that the ACs had only reached the jejunum but not the ileum 2 h after administration. These results suggested that the mixture of BCAs and IP_6 reduced gastrointestinal motility, especially in the stomach and jejunum. Our data indicated the prolongation of ACs residues in the gastrointestinal tract was caused by IP_6 enhancing absorption of AC. There are no reports of IP_6 reducing gastrointestinal motility (H. Ishikawa, personal communication, 2006), and it appears that only the combination of ACs and IP_6 is effective for reducing gastrointestinal motility.

ACs are absorbed and eliminated extremely rapidly and are absorbed with poor efficiency as compared to other polyphenols. Several recent reports have identified the absorption site of ACs, with Talavéra et al. demonstrating that a high proportion of anthocyanin monoglycosides was absorbed from the stomach. This absorption was dependent on anthocyanin structure with delphinidin glycosides being the most readily absorbed (*22*). Passamonti also showed in an in vivo experiment in rats that the stomach was involved in the absorption of grape anthocyanins (*23*). In the present study, the AC contents in the stomach were increased and remained at this level in the IP_6 group. If it is assumed the main site of absorption of ACs is the stomach, this would provide considerable support to the scientific rationale of our study.

Talavéra et al. also showed that anthocyanin glycosides were rapidly and efficiently absorbed from the small intestine (jejunum + ileum) in a rat in situ study (*24*), while Matuschek et al. found in an in vitro study in mice that absorption of AC occurred predominantly in the jejunum as compared to the duodenum, ileum, or colon (*25*). These reports also supported the findings of our study in that the presence of ACs in the jejunum occurred earlier than in either the plasma or the urine and corresponded to the plasma concentration and urinary excretion of ACs. Ileum may be a main site of anthocyanin absorption. In our study, although the ACs of ileum are high in the control group, the plasma concentration and urinary excretion of ACs are quite small in the control group.

We also measured the urinary recovery rate of ACs in the 24-h period following administration of either elderberry and bilberry ACs administration in rats. The urinary recovery rates in the 24 h after administration of these ACs without IP_6 were 0.1−0.3%, a recovery rate similar to that of BCA. In rats administered a combination of IP_6 and ACs from either berry, the AC absorption enhancing effect of IP_6 was also apparent, being 4.3 times greater for elderberry ACs and 10.6 times greater for bilberry ACs. These results suggested that the enhancing effect of IP_6 on AC absorption was effective not only for BCAs, but also for other berry ACs.

In our human study, the plasma AC concentrations in the subjects showed moderate variability. The concentrations were almost less than 1 ng/mL in the control group, whereas in the BCA-IP_6 group, the plasma AC concentrations in all of the subjects were markedly increased. The mean plasma AC concentration was always higher in the BCA-IP_6 group, a finding consistent with the results obtained from our studies in rats.

The excretion time of ACs was variable in the subjects in both the control and the BCA-IP_6 beverage drinking groups, with the urinary excretion of ACs in all subjects in the BCA-IP_6 group being markedly increased. Comparison of the mean amount of ACs excreted showed the BCA-IP_6 group always had the highest level of excretion. Moreover, the peak of AC excretion was delayed until 4−8 h post-administration, and the recovery of AC in the IP_6 group was 4.5 times higher than that without IP_6. These results were in agreement with the results from our studies in rats.

In the present study, the four ACs, D3R, C3R, D3G, and C3G, were detected mainly in both plasma and urine as intact forms following oral administration of BCAs. In our recent study, three purified ACs, D3R, C3R, and C3G, and BCAs were administered orally to both rats and humans. This study showed that these compounds were absorbed and became distributed mainly in the blood and excreted in the urine as intact forms (*9*). We reported the detection of small amounts of the metabolite 4′-*O*-methyl-delphinidin-3-rutinoside in plasma, but did not detect either anthocyanidin (aglycone) or glucuro- or sulfoconjugates. Other studies have reported that several metabolites are formed after oral ingestion of ACs. For example, glucuronyl is formed from cyanidin-3-glucoside and both glucuronyl and sulfate conjugates from pelargonidin-3-glucoside. Our results indicate that delphinidin-3-rutinoside may be metabolized differently from cyanidin-3-glucoside and pelargonidin-3-glucoside. In our comparative study of IP_6 and phytin in rats, only the IP_6 group exhibited an AC absorption enhancing effect. This result suggested that the chelating action of IP_6 may participate in this enhancement of AC absorption.

The molecular weights of anthocyanin glycosides are usually approximately 400−600 daltons. However, in aqueous solution, ACs exist as high molecular complexes with other polyphenols, metal ions, carbohydrates, and organic acids. For example, a blue pigment complex, protodelphin, was isolated from the blue flowers of *Salvia patens* (*26*). Protodelphin was shown to be composed of delphinidin 3-(6″-*p*-coumaroylglucoside)-5-(6″-malonylglucoside), apigenin 7,4′-diglucoside, and magnesium. Similar blue complexes manifested as metallo-anthocyanins are formed with iron, manganese, cobalt, nickel, zinc, and cadmium instead of magnesium.

The mechanism by which IP_6 enhances AC absorption remains unresolved. However, as phytin has no such effect on AC absorption, it is possible that the strong chelating effect of IP_6 on metal ions, such as iron and magnesium, contributes to the enhanced AC absorption. It is also possible that IP_6 may transform from a huge molecular group to another situation by chelating metal ion, thereby tending to remain in the stomach and jejunum. The mechanism of AC absorption from the stomach was also not established. Walgren et al. reported that AC absorption in the small intestine involved an interaction with the intestinal sodium-dependent glucose transporter, SGLT1 (*27*), although the absorptive mechanism was also not elucidated in detail.

One limitation of this study is the small number of humans in the design of human study. It is necessary to further a large study to confirm the IP_6 enhancement of AC absorption and to set the suitable IP_6 dose in human. To demonstrate the mechanism involved in IP_6 enhancement of AC absorption, it will be necessary to perform further studies on the mechanism of absorption of high molecular ACs in aqueous solution. As our data showed the intake of commercial blackcurrant juice with IP_6 increased the plasma level and urinary excretion of AC, this raised the possibility of using IP_6 as an enhancer of

AC absorption. Under Japanese food sanitation law, the use of IP_6 is permitted as an acidulant with no upper limitation on dosage. Although several safety studies have conformed to these sanitation laws, there have been no studies on the intake of high doses of IP_6 in humans. Safety tests are therefore necessary before high dose IP_6 can be used in foods. As a consequence of the reduction in the stomach motility caused by intake of BCAs and IP_6, subjects may feel their stomachs "feeling heavy". Yet, throughout the study, all of the subjects did not report these side effects.

In conclusion, the present studies in rats demonstrated that orally administered IP_6 with AC dramatically enhanced AC absorption to the plasma and excretion in the urine. This AC absorption enhancing effect of IP_6 was also confirmed in human plasma and urine. The enhancement of AC absorption by the addition of IP_6 may prolong the transient time by decreasing gastrointestinal motility. This is the first report to show specifically that a combination of foods enhances the absorption of ACs.

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