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Direct Absorption of Acylated Anthocyanin in Purple-Fleshed Sweet Potato into Rats

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Absorption of acylated anthocyanins in purple-fleshed sweet potato (*Ipomoea batatas* cv. Ayamurasaki) in rats was studied to obtain evidence that the acylated anthocyanins themselves could exert a physiological function in vivo. Peonidin 3-caffeoylsophoroside-5-glucoside (Pn 3-Caf•sop-5-glc) in purple-fleshed sweet potato was directly absorbed into rat and present as an intact acylated form in plasma. After oral administration of the purple-fleshed sweet potato anthocyanin (PSA) concentrate containing 38.9 μmol of Pn 3-Caf•sop-5-glc/kg of body weight, Pn 3-Caf•sop-5-glc was detected in the plasma, and the C_{max} value and t_{max} were estimated as 50.0 $\pm$ 6.8 nmol/Lof plasma and 30 min, respectively. Furthermore, the plasma antioxidant capacity was significantly elevated from 58.0 $\pm$ 12.0 to 89.2 $\pm$ 6.8 μmol of Trolox equivalent/L of plasma 30 min after the administration of the PSA concentrate.

KEYWORDS: Anthocyanin; absorption; plasma antioxidant capacity; sweet potato

INTRODUCTION

Anthocyanins are widely distributed in fruits, beans, cereals, vegetables, and red wines, and humans ingest a considerable amount of anthocyanins in plant-based daily diets. In the past decade, anthocyanin-rich foods and preparations have attracted attention due to their health-promoting benefits in terms of reducing the risk of coronary heart disease and preventing some chronic diseases. For example, the "French paradox" (a low incidence of coronary heart disease and atherosclerosis despite a high-fat diet) of red wine (*1*), the ophthalmic activity of bilberry extract (*2*), and the hepatic function restorative activity of purple-fleshed sweet potato juice (*3*, *4*) are well-known. With such information, the attention of researchers has been focused recently on the potential role of anthocyanins in vivo, including their absorption, metabolic fate, and physiological function.

There have been several quantitative studies on the absorption of anthocyanins, for example, cyanidin 3-glucoside (C3G) (*5*−*7*), cyanidin 3,5-diglucoside (*6*), cyanidin 3-rutinoside (C3R) (*7*), and delphinidin 3-rutinoside (D3R) (*7*). A few reports demonstrated that anthocyanins in *Vaccinium myrtillus* (*8*) and wine (*9*) are absorbed by the body after ingestion. However, there have been few reports on the absorption of acylated anthocyanins, although many plant anthocyanins are present as acylated forms.

The purple-fleshed sweet potato (*Ipomoea batatas*) contains a high level of anthocyanins, and their contents vary with sweet potato variety (*10*). The Ayamurasaki cultivar contains anthocyanins of 0.59 mg of peonidin 3-caffeoylsophoroside-5-glucoside (Pn 3-Caf•sop-5-glc) equivalent/g (*10*). Its predominant anthocyanin pigments were identified as mono- or diacylated forms of cyanidin and peonidin by HPLC-MS (*11*−*13*). Furthermore, HPLC analysis showed that anthocyanins in the Ayamurasaki cultivar are composed of acylated peonidin (74%), acylated cyanidin (19%), and others (7%) (*14*). The chemical structure of acylated peonidin is shown in **Figure 1**. On the other hand, the extract of purple-fleshed sweet potato possesses antioxidative or radical scavenging (*10*), antimutagenic (*15*, *16*), and angiotensin I-converting enzyme (ACE) inhibiting (*17*) properties. In a rat study, an ameliorative effect against carbon tetrachloride-induced liver injury was exhibited in rats administered the purple-fleshed sweet potato juice but not in rats administered the orange-fleshed sweet potato juice (*3*). In addition, the juice (44 days of continuous ingestion of anthocyanins of 23.7 mg of Pn 3-Caf•sop-5-glc equivalent/day) has abilities to restore to normal levels of serum γ-GTP, GOT, and GPT in human volunteers with impaired hepatic function and to reduce to normal levels of blood pressure in volunteers with

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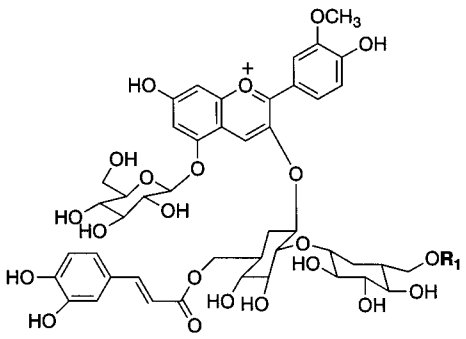

Abbreviations	R_1
Pn 3-diCaf·sop-5-glc	caffeic acid
Pn 3-Caf·pHB·sop-5-glc	*p*-hydroxy benzoic acid
Pn 3-Caf·sop-5-glc	H
Pn 3-Caf·Fr·sop-5-glc	ferulic acid

Pn, peonidin; Caf, caffeic acid;
pHB, *p*-hydroxy benzoic acid; Fr, ferulic acid
sop, sophorose; glc, glucose

Figure 1. Chemical structures of major anthocyanins in purple-fleshed sweet potato.

hypertension (*4*). These findings strongly suggested that the acylated anthocyanins in purple-fleshed sweet potato might exert a physiological function in vivo. However, we would not say with certainty that the acylated anthocyanins exert a physiological function in vivo because there are no reports stating that the chemical can be absorbed into the body.

In this study, we prepared a purple-fleshed sweet potato anthocyanin (PSA) concentrate and investigated the absorption of mono- and diacylated forms of anthocyanins contained in PSA, especially the major component, Pn 3-Caf·sop-5-glc, into rat plasma. In addition, we tried to verify whether the plasma antioxidant capacity improved after oral administration of the PSA concentrate.

MATERIALS AND METHODS

Chemicals. A water-soluble tocopherol analogue, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), was obtained from Aldrich Chemical Co. (Milwaukee, WI). The reagents used for the chemiluminescence assay, the ECL Western blotting detection reagent (RPN2106), and horseradish peroxidase (HRP) conjugate (mouse Ig, HRP-labeled whole antibody, from sheep) were purchased from Amersham Pharmacia Biotech Japan (Tokyo, Japan). Other reagents were of analytical grades and used without further purification.

Preparation of Purple-Fleshed Sweet Potato Anthocyanin Concentrate. Purple-fleshed sweet potato (*I. batatas* cv. Ayamurasaki) was harvested in Miyazaki prefecture in 1998. Tubers were washed with tap water, peeled, and heated at 90 °C for 10 min. The heated flesh was ground in the same weight of water by using a Super Masscolloider (Masuko Sangyo Co.) and centrifuged. The resultant sweet potato juice was treated with a composed-enzyme solution containing amylase, cellulase, hemicellulase, and pectinase at 50 °C for 60 min and centrifuged. The supernatant was concentrated by a continuous evaporator and stored at −20 °C until use.

The concentrated sweet potato extract, 2.8 kg (containing anthcyanins of 2.9 mg of Pn 3-Caf·sop-5-glc equivalent/g), was acidified in 4 volumes of 1% acetic acid solution and put on a Diaion HP-20 (Nippon Rensui Co., Tokyo, Japan) column (10 cm i.d. × 100 cm) directly. After washing with 15 L of distilled water, the anthocyanin fraction was eluted with 5 L of a 70% ethanol solution. The ethanolic elute was concentrated to 18 °Brix under reduced pressure at 35 °C and then freeze-dried. The obtained PSA concentrate contained anthocyanin at a concentration of 124.4 mg of Pn 3-Caf·sop-5-glc equivalent/g, calculated with a molar extinction coeffcient of $2.76 \times 10^4\ M^{-1}\ cm^{-1}$ for Pn 3-Caf·sop-5-glc in 1% trifluoroacetic acid (TFA) solution. In this PSA concentrate, chlorogenic acid was also contained in the concentration of 22.8 mg/g; however, ascorbic acid and tocopherols were not detected.

Isolation of Acylated Anthocyanins from Purple-Fleshed Sweet Potato. Pn 3-Caf·sop-5-glc and other acylated anthocyanins were isolated as TFA salts from purple-fleshed sweet potato by HP-20 column chromatography, LH-20 column chromatography, preparative paper chromatography, preparative TLC, and preparative HPLC as described previously (*12*, *13*). The purity of Pn 3-Caf·sop-5-glc was >95.0% by HPLC analysis.

Animals and Oral Administration of PSA Concentrate. Six-week-old male Wistar rats were obtained from CLEA Japan Ltd. (Tokyo, Japan). All rats were kept in an air-conditioned room (23 ± 1 °C and 55 ± 5% humidity) under a 12-h dark/light cycle with free access to tap water and commercial diet (type CE-2, CLEA Japan) for a week. Rats ($n = 20$) were fasted for 24 h before administration and were randomly divided into five groups. The PSA concentrate was dissolved in distilled water at a concentration of 29.3 mg of Pn 3-Caf·sop-5-glc equivalent/mL (this solution contains 7.8 μmol of Pn 3-Caf·sop-5-glc/mL by HPLC analysis) and administrated orally to rats by direct stomach intubation at a dosage of 146.3 mg of Pn 3-Caf·sop-5-glc equivalent/kg of body weight (containing 38.9 μmol of Pn 3-Caf·sop-5-glc/kg). Before (control) and 15, 30, 60, and 120 min after administration, all rats were anesthetized with diethyl ether; blood was collected by heart puncture at each time point into heparinized tubes (type VP-AL076, Terumo, Tokyo, Japan), and plasma was prepared by centrifugation at 1600*g* for 15 min at 4 °C. During the experiments, all animals were handled in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (NRC 1985).

Determination of Pn 3-Caf·sop-5-glc in Plasma. The extraction of anthocyanins from plasma was done according to the method of Miyazawa et al. (*6*) with a slight modification. The plasma (1 mL) was acidified with 200 μL of 5% TFA solution, and the mixture was subjected to a Sep-Pak C18 cartridge (Waters, Milford, MA). After washing with 7% formic acid solution (10 mL), dicholoromethane (15 mL), and benzene (15 mL), the cartridge was dried in N_2 gas. Acylated anthocyanins were eluted with 5 mL of methanol containing 5% TFA. The eluate was evaporated to dryness in vacuo at 35 °C. The dried extract was redissolved in 200 μL of 15% acetonitrile in water containing 1.5% phosphoric acid. An aliquot (100 μL) of this solution was subjected to a Wakosil II-AR (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) column (4.6 mm i.d. × 250 mm) with a flow rate of 0.75 mL/min by an isocratic elution (1.5% phosphoric acid, 15% acetonitrile in water). Anthocyanins were monitored at 530 nm while a UV−vis spectrum was obtained by an MD-1510 photodiode array (JASCO Co., Ltd., Tokyo, Japan). The detection limit of Pn 3-Caf·sop-5-glc was about 550 fmol/injection judging from the signal to noise ratio (S/N) of >3. When 46.0 pmol of Pn 3-Caf·sop-5-glc was added to 1 mL of rat plasma, the recovery of Pn 3-Caf·sop-5-glc was 75.9 ± 3.3% (means ± SD of three experiments).

Measurement of Plasma Antioxidant Capacity. Plasma antioxidant capacity was measured by a modification of the enhanced chemiluminescence assay used in the study on serum antioxidant capacity after red wine ingestion (*18*, *19*). Plasma was prepared from the collected blood by centrifugation, immediately diluted with the same volume of deaerated 10 mM oxalic acid, stored at 4 °C in the dark, and processed within 2 h in the following manner. The sample (40 μL) was then diluted with 160 μL of distilled water, and 20 μL (equivalent to 2 μL of plasma) was added into the following assay system. The assay mixture consisted of phosphate-buffered saline (1000 μL, pH 7.4), reagent A (40 μL), HRP-labeled antibody solution (20 μL) diluted with 8000 volumes of phosphate-buffered saline containing 0.1% Tween 20, the diluted plasma sample or Trolox solution (20 μL), and distilled water (520 μL). The reaction was initiated by the addition of 400 μL of reagent B diluted one-tenth with distilled water. Here, reagents A and B were ECL detection reagents. The chemiluminescence was measured by a chemiluminescence analyzer (type CLD-110, Tohoku Electronic Industrial Co., Sendai, Japan) at an interval of 10 s for 4 min. Because the sum of light emission for 4 min was decreased linearly

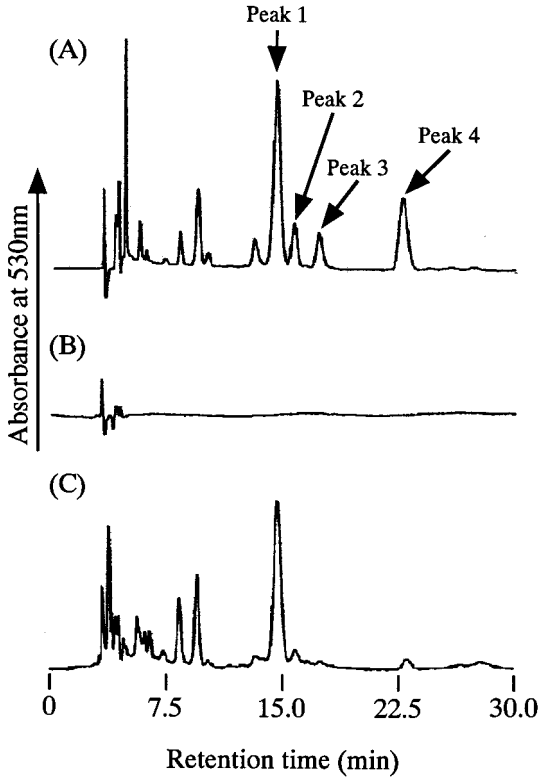

Figure 2. HPLC chromatograms of PSA concentrate (A) and anthocyanins detected in rat plasma before (B) and after (C) PSA administration. Peak 1, Pn 3-Caf·sop-5-glc; peak 2, Pn 3-diCaf·sop-5-glc; peak 3, Pn 3-Caf·pHb·sop-5-glc; peak 4, Pn 3-Caf·Fr·sop-5-glc.

in proportion to the Trolox concentration, the plasma antioxidant capacity was calculated from the calibration curves for Trolox.

Statistics. All data are reported as means ± SD (standard deviation). The statistical significance was evaluated using Student's *t* test, and $p < 0.05$ was taken as significant.

RESULTS

Direct Absorption of Acylated Anthocyanin in Rat. Typical HPLC profiles of anthocyanins detected in rat plasma before and after administration of the PSA concentrate are shown in **Figure 2**. Many peaks were observed in the chromatogram of plasma after administration of the PSA concentrate, but there were no peaks in rat plasma before administration. These peaks were presumed to be the anthocyanins derived from PSA due to their similarity with the chromatogram of the PSA concentrate. A major peak detected in rat plasma (at 14.5 min of retention time) was identified as Pn 3-Caf·sop-5-glc by comparison with the retention time and UV−vis spectrum of authentic Pn 3-Caf·sop-5-glc. **Figure 3** shows the time course changes in the concentrations of Pn 3-Caf·sop-5-glc in rat plasma after administration of the PSA concentrate. The plasma concentration of Pn 3-Caf·sop-5-glc reached a maximum of 50.0 ± 6.8 nmol/L of plasma at 30 min postadministration and then decreased gradually. The pharmacokinetic parameters calculated from the values shown in **Figure 3** were as follows: the $t_{1/2}$ value and area under concentration (AUC) were estimated as 0.68 h and 46.9 nmol·h/L of plasma, respectively.

Antioxidant Capacity in Rat Plasma after PSA Administration. The plasma antioxidant capacity before or 30 min after administration of the PSA concentrate was measured. **Figure 4** shows the typical light emission kinetics obtained from rat plasma. As compared to control rat plasma, the sum of light emission for 4 min was lower in plasma at 30 min postadministration of the PSA concentrate, indicating a higher antioxidant capacity in plasma 30 min after PSA administration. When plasma antioxidant capacity was calculated by using Trolox, the plasma antioxidant capacity after PSA administration was significantly elevated ($p < 0.05$) from 58.0 ± 12.0 to 89.2 ± 6.8 μmol of Trolox equivalent/L of plasma (**Figure 5**).

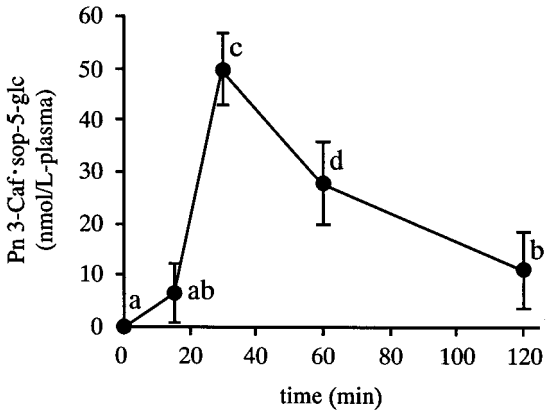

Figure 3. Time course changes in Pn 3-Caf·sop-5-glc concentration in rat plasma after PSA administration. Values are means ± SD of four rats. Means with different letters are significantly different at $p < 0.05$.

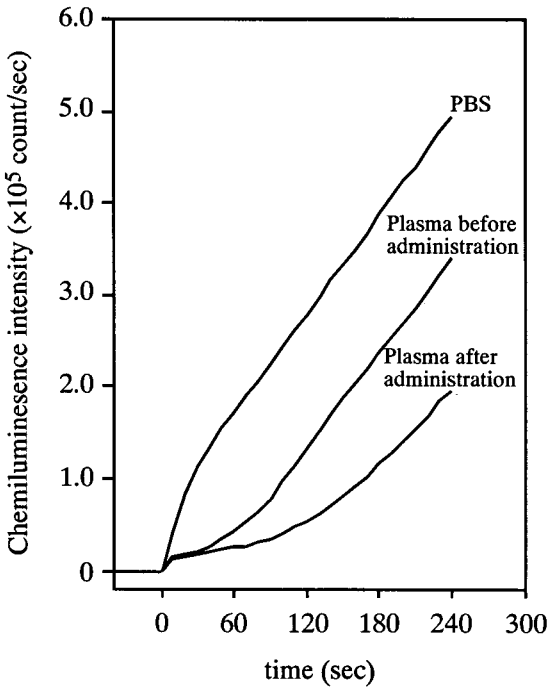

Figure 4. Typical light emission kinetics obtained from rat plasma before and after PSA administration.

DISCUSSION

In our series of studies concerning the physiological functions of purple-fleshed sweet potato (*3*, *4*), we speculated that anthocyanins might be major contributors in vivo due to their potent activities in vitro as radical scavengers (*10*) and ACE inhibitors (*17*). However, we were not confident because there was a lack of evidence for the absorption of acylated anthocyanins contained in purple-fleshed sweet potato. In this study, we confirmed that one of the acylated anthocyanins in purple-fleshed sweet potato, Pn 3-Caf·sop-5-glc, was absorbed directly into rats. Other diacylated anthocyanins, Pn 3-diCaf·sop-5-glc, Pn 3-Caf·pHb·sop-5-glc, and Pn 3-Caf·Fr·sop-5-glc, were

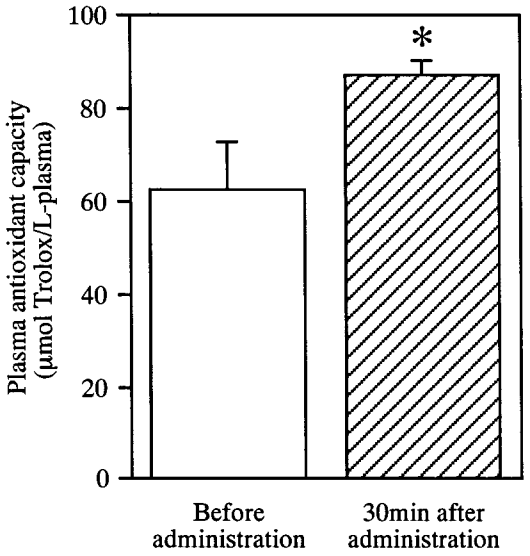

Figure 5. Elevated plasma antioxidant capacity after PSA administration. Values are means ± SD of four rats. Asterisk indicates significant difference ($p < 0.05$) from control value (before administration).

probably absorbed as well; however, their concentration in plasma was very low for quantitative analysis. The molecular weight of Pn 3-Caf·sop-5-glc (MW = 949) was larger than those of C3G (MW = 449), cyanindin-3,5-diglucoside (MW = 611), D3R (MW = 611), and C3R (MW = 595), which have already been reported to be absorbed into rats or humans. Thus, our results indicate that there would be other types of anthocyanins able to be absorbed into animals within the range of acylated anthocyanins and anthocyanins with MW = 950.

It is well-known that the absorption of anthocyanins or flavonoids is influenced by a difference in structural features (*7*). The time course of changes in the concentrations of Pn 3-Caf·sop-5-glc showed an estimated t_{max} value of 30 min (**Figure 3**). The value was similar to the t_{max} value of 30 min observed in C3G and C3R, which would be incorporated through the hexose transport pathway (*7*). This indicated that the speed of absorption was not influenced even when anthocyanin was acylated.

It is of interest that Pn 3-Caf·sop-5-glc was detected in the plasma in an intact form. In an in vitro study, Pn 3-Caf·sop-5-glc exhibited potent antioxidative activity as well as Cy 3-Caf·Fr·sop-5-glc, Pn 3-Caf·pHb·sop-5-glc, and Pn 3-Caf·Fr·sop-5-glc (*20*). Thus, it can clearly be postulated that Pn 3-Caf·sop-5-glc may exert its antioxidative activity in vivo. This postulation was supported by the result that rat plasma after the administration of PSA concentrate showed 1.5 times higher antioxidant activity than that before administration. The fact that Pn 3-Caf·sop-5-glc was present in plasma with a trace of other acylated anthocyanins also suggested the possibility that the absorbed acylated anthocyanins might have contributed to lowering blood pressure in volunteers with hypertension (*4*) through the inhibition against ACE in the circulatory system and tissues or to preventing of liver injury in rats (*3*) through the suppression of the trichloromethyl radical.

In conclusion, we demonstrated that the major acylated anthocyanin (Pn 3-Caf·sop-5-glc) in purple-fleshed sweet potato was absorbed directly into rat plasma and detected in an intact form. This is the first evidence of direct absorption of mono- and diacylated anthocyanins. In addition, it was clarified that plasma antioxidant capacity was significantly elevated after the ingestion of PSA concentrate.

ABBREVIATIONS USED

Cy 3-Caf·Fr·sop-5-glc, cyanidin 3-caffeoylferuloylsophoroside-5-glucoside; Pn 3-Caf·Fr·sop-5-glc, peonidin 3-caffeoylferuloylsophoroside-5-glucoside; Pn 3-Caf·pHB·sop-5-glc, peonidin 3-caffeoyl-*p*-hydroxybenzoylsophoroside-5-glucoside; Pn 3-Caf·sop-5-glc, peonidin 3-caffeoylsophoroside-5-glucoside; Pn 3-diCaf·sop-5-glc, peonidin 3-dicaffeoylsophoroside-5-glucoside.

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