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Report

Effect of Acylated Sugar Chain Terminals on the Intestinal Absorption of Large Molecular, Heavenly Blue Anthocyanin in Rats

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The gastrointestinal absorption of heavenly blue anthocyanin derived from morning glory (*Pharbitis nil* L.) was examined in rats. Ingested heavenly blue anthocyanin was directly absorbed from the gastrointestinal tract and detected in its original polyacylated form in rat blood plasma. The maximum plasma concentration and area under the plasma concentration curve during 8-h post-administration after a single oral dose of 0.0569 mmol/kg heavenly blue anthocyanin were 0.143 ± 0.023 μM and 23.30 ± 3.76 μM·min, respectively. Heavenly blue anthocyanin, which contains asymmetrical branched chains with glucosyl terminals, was absorbed at a similar level to that of total ternatin. By contrast, the plasma amount of heavenly blue anthocyanin was approximately 6-fold higher than that in a previous report of peonidin 3-*O*-β-(6″-*O*-caffeoyl)-sophoroside-5-*O*-β-D-glucopyranoside with an attached caffeoyl terminal on 6″-position.

Key words heavenly blue anthocyanin, morning glory, peonidin 3-*O*-β-(6″-*O*-caffeoyl)-sophoroside-5-*O*-β-D-glucopyranoside, intestinal absorption, terminal structure

INTRODUCTION

Anthocyanins are a family of flavonoids widely distributed in plants[1] and their biological functions have attracted much attention.[2] Acylated anthocyanins are stable analogs of anthocyanin because of intramolecular hydrophobic stacking between the anthocyanidin (aglycon of anthocyanin) and aromatic rings on the acyl moieties.[3] In addition, the side chain acyl groups consisting of acylated anthocyanins are expected to contribute positively to their biological functions.[4]

Studies on the gastrointestinal absorption of acylated anthocyanins to prove their *in vivo* functions have been reported.[5,6] We previously found that typical acylated anthocyanin, nasunin (delphinidin 3-*O*-(6-*O*-(4-*O*-*p*-coumaroyl-α-L-rhamnopyranosyl)-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside), was absorbed from the gastrointestinal tract to a similar extent as non-acylated anthocyanin, delphinidin 3-*O*-β-D-glucopyranoside,[6] despite its large molecular weight of 919. Furthermore, we recently discovered that ternatins, derived from the petals of butterfly pea (*Clitoria ternatea* L.) encompassing of molecular weight range of 1330 to 2109, were absorbed at a similar level to those of non-acylated anthocyanins.[7] In contrast, peonidin 3-*O*-β-(6″-*O*-caffeoyl)-sophoroside-5-*O*-β-D-glucopyranoside (Pn3CafSop5G), the dominant anthocyanin in purple sweet potato (*Ipomoea batatas* L.), showed a specifically low absorption in rats.[5]

Heavenly blue anthocyanin (HBA) is a polyacylated anthocyanin found in the petals of morning glory (*Pharbitis nil* L.) (MGP).[8] The structure of HBA was elucidated to be peonidin 3-*O*-(2″-*O*-(6‴-*O*-(3-*O*-(β-D-glucopyranosyl)-caffeoyl)-β-D-glucopyranosyl)-6″-*O*-(4-*O*-(6-*O*-(3-*O*-(β-D-glucopyranosyl)-caffeoyl)-β-D-glucopyranosyl)-caffeoyl)-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside.[9] HBA and Pn3CafSop5G contain peonidin 3-*O*-β-(6″-*O*-caffeoyl)-sophoroside-5-*O*-β-D-glucopyranosyl part as a common structure, and HBA has two more chains consisting of 6″-*O*-(4-*O*-(6-*O*-(3-*O*-(β-D-glucopyranosyl)-caffeoyl)-β-D-glucopyranosyl)-caffeoyl)-β-D-glucopyranosyl (–CafG-CafG) and 6‴-*O*-(3-*O*-(β-D-glucopyranosyl)-caffeoyl)-β-D-glucopyranosyl (–CafG) on the sophorose residue (Fig. 1). HBA has Pn3CafSop5G as a partial structure and its acylated sugar moieties substitution positions and aromatic acid residues is different compared with ternatins; therefore, we investigated the gastrointestinal absorption of HBA in rats to determine how the substitution patterns of the branched chains influence acylated anthocyanin bioavailability.

MATERIALS AND METHODS

Reagents HPLC-grade acetonitrile was obtained from Kanto Chemical Co., Ltd. (Tokyo, Japan). All other reagents, including trifluoroacetic acid (TFA), were purchased from Wako Pure Chemical Industry (Osaka, Japan) and used without further purification. HP–20 resin was purchased from Mitsubishi Chemical Co. (Tokyo, Japan). Sephadex LH–20 (25–100 μm) was obtained from Amersham Biosciences, Inc. (Buckinghamshire, UK).

Plant Material Morning glory (*Pharbitis nil* L.) was

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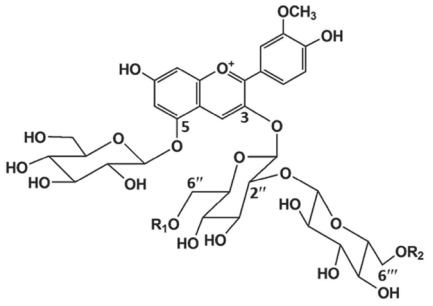

Anthocyanin	R_1	R_2	Molecular weight
HBA	–CafGCafG	–CafG	1760
Pn3CafSop5G	–Caf	–H	949

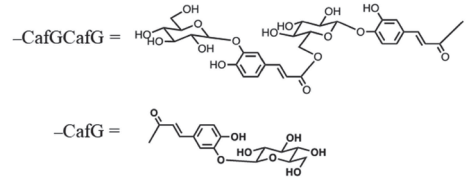

Fig. 1. Structures of HBA and Pn3CafSop5G

Caf, caffeoyl; G, D-glucosyl residue.

grown on a farm at Minami-Kyushu University and the flower petals were collected during July and October 1994, dried at 45°C overnight, and stored in a silica gel desiccator until used for extraction.

Preparation of MGP Extract MGP extract was obtained as previously described with modifications.[8] Briefly, dried MGP (100 g) was immersed in 6 L of 1% acetic acid containing 90% aqueous methanol at room temperature for 5 h. The extract was concentrated to dryness *in vacuo*. MGP extract containing HBA (prepared above) was adsorbed on an HP–20 resin column (60 × 450 mm), washed with 1% acetic acid aqueous, and the anthocyanin fraction was eluted with 70% aqueous ethanol containing 1% acetic acid. After concentration, the residue was dissolved in 1% acetic acid aqueous and chromatographed on an LH–20 column (45 × 100 mm) in the same solvent and the anthocyanin fraction was eluted with 70% aqueous ethanol containing 1% acetic acid and concentrated to dryness *in vacuo*. The amount of extract recovered was 0.8 g.

Purification of HBA The MGP extract prepared above was dissolved in 15% acetic acid aqueous and HBA in the extract was isolated by semi-preparative HPLC using an Inertsil ODS-2 column (20 × 250 mm) under isocratic elution conditions.[8] The mobile phase was a mixture of solvent A (15% acetic acid aqueous) and solvent B (15% acetic acid containing 30% acetonitrile aqueous) at various ratios (65%A/35%B to 90%A/10%B (v/v)), with a flow rate of 7 mL/min, and the effluent was monitored at 530 nm. After the eluent was concentrated to dryness, the isolated pigment was dissolved in a small amount of TFA and precipitated with excess diethyl ether to obtain TFA salts as a reddish-purple powder. The pigment structure was identified by FAB–MS and NMR spectrometry, as described previously.[8] The purity of HBA was > 94.4% calculated from the peak area of the HPLC chromatogram.

HPLC Analysis HPLC conditions were described in our previous report with modification.[7] Briefly, aliquots (100 µL) of sample solutions were injected into an HPLC system (Hitachi L-7200) equipped with a Develosil ODS-HG 5 column (1.0 × 150 mm) using 0.5% TFA aqueous (solvent A) and 0.5% TFA containing acetonitrile (solvent B) in linear gradient elution. The gradient conditions were as follows: 87.5% A/12.5% B (v/v) to 73.5% A/26.5% B (v/v) for 40 min, and 55% A/45% B (v/v) for 20 min at a flow rate of 0.1 mL/min. The elution profile was monitored at 530 nm with a UV–VIS detector (Hitachi L-7000).

Animals and Diets SPF male Wistar ST rats (5-weeks-old, bodyweight of 160 g) purchased from Japan SLC, Inc., (Hamamatsu, Japan) were individually housed in stainless-steel wire-mesh cages at 23 ± 1°C for conditioning under a 12-h light/dark cycle. Rats were allowed *ad libitum* access to tap water and a controlled diet for 7 d before the experiment. Animal experiments were performed in accordance with the Guidelines on the Care and Use of Laboratory Animals issued by Niigata University of Pharmacy and Applied Life Sciences. The protocol was approved by the Committee on the Ethics of Animal Experiments of Niigata University of Pharmacy and Applied Life Sciences (Approval number 17-4). All efforts were made to minimize suffering.

Experimental Design After 7 d of adaptation, three rats were cannulated with a polyethylene tube (PE 50) into a neck vein under anesthesia with a mixed solution of medetomidine hydrochloride (0.15 mg/kg), midazolam (2 mg/kg), and butorphanol tartrate (2.5 mg/kg) by intraperitoneal injection as we described previously with modifications.[7] Briefly, the neck vein was isolated, and a small hole cut with scissors to insert the PE 50. Once the tube was inserted, the vein and tube were occluded, and the tube was guided out from the back of the rats. After starving for 24 h, purified HBA dissolved in 0.1% aqueous citric acid was administered orally (100 mg of purified HBA/kg body weight) to three rats, as we previously described.[7] During the experiment, the rats were allowed to move freely in the cages. Blood samples were collected *via* the cannulated tube using a heparinized syringe at 0, 15, 30, 60, 120, 240, and 480 min. Donor blood was collected from other healthy rats under anesthesia by venipuncture of the inferior vena cava into a sodium citrate solution (500 µL of 10% sodium citrate per 8 mL of blood). After the blood was withdrawn (600 µL), the same volume of donor blood was injected through the cannulated vein tube. Each blood sample was immediately centrifuged at 3000 × *g*, 4°C for 5 min to prepare plasma samples for HPLC analysis.

Plasma Preparation HBA was extracted from blood plasma using a Sep-Pak C_{18} Light cartridge (Waters, Milford, MA, USA) as we previously reported.[7] Briefly, plasma (300 µL) samples were applied to the Sep-Pak C_{18} Light cartridges conditioned with methanol (2 mL) and 3% aqueous TFA solution (2 mL). The cartridges were then washed successively with 2 mL of 3% aqueous TFA, dichloromethane, and benzene, and HBA was eluted with 50% aqueous acetonitrile containing 1% TFA. The eluent was concentrated to dryness *in vacuo* and dissolved in 150 µL of 0.5% aqueous TFA. The dissolved sample was passed through a Centricut ultra-membrane filter (0.45 µM; Kurabou Co. Ltd., Osaka, Japan) before HPLC injection. Recovery of the sample by this method was 88.7%, as verified by spiking purified HBA into normal blood plasma.

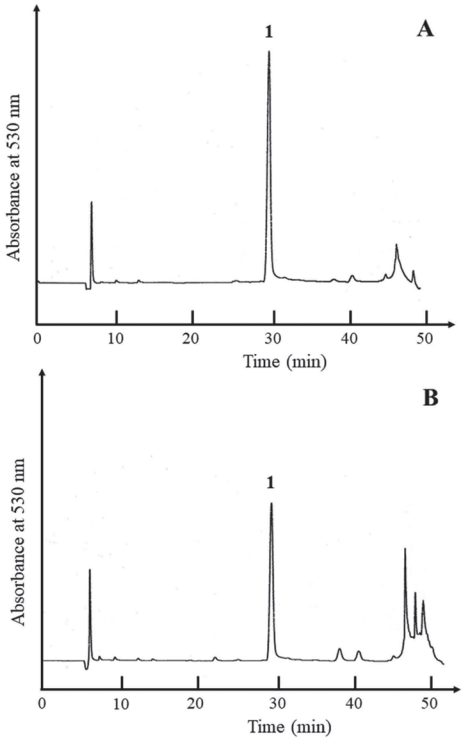

Fig. 2. Typical HPLC Chromatogram of HBA and Rat Blood Plasma

A: Purified HBA. **B**: Rat blood plasma 15 min after the oral administration of purified HBA. Peak 1 corresponds to HBA.

RESULTS AND DISCUSSION

Figure 2A shows a typical HPLC chromatogram of purified HBA detected at 530 nm. HPLC analysis revealed that HBA was absorbed from the gastrointestinal tract and present in rat blood plasma at 15 min after the oral administration of HBA at a dose of 100 mg/kg (0.0569 mmol/kg) (Fig. 2B). Several peaks which eluted later than HBA were also detected in HPLC chromatogram of rat blood plasma (Fig. 2B). These peaks might be the metabolites of HBA produced *in vivo*, although, no structural information was obtained. The maximum plasma concentration (C_{max}) of HBA was 0.143 ± 0.014 μM at 30 min after oral administration, and the area under the plasma concentration curve during 8 h after oral administration (AUC_{0-8}) obtained from the plasma concentration profile of HBA was 23.30 ± 3.76 μM·min (Fig. 3). The relative standard errors versus mean values of HBA obtained from three rats were 16.13% for AUC_{0-8} and 9.43% for C_{max}. These levels were similar to those for ternatins absorption we recently reported (5.46 to 15.93% for AUC_{0-8} and 3.01 to 32.28% for C_{max}).[7]

Because we investigated whether a large molecule, HBA, was directly absorbed from the gastrointestinal tract, the intravenous administration of HBA was not performed in the present study, and pharmacokinetic parameters including volume of distribution were not obtained. Instead of these parameters, AUC_{0-8} and C_{max} values were normalized to the orally administered dose (AUC_{0-8}/D and C_{max}/D) to compare the gastrointestinal absorption of acylated anthocyanins. The C_{max}/D (2.51 ± 0.23 μM/mmol/kg) and AUC_{0-8}/D (409.51 ± 66.03 μM·min/mmol/kg) values of HBA were similar to those of total ternatin previously reported: 2.68 ± 0.67 μM/mmol/kg for C_{max}/D and 311.15 ± 29.26μM·min/mmol/kg for AUC_{0-8}/D.[7]

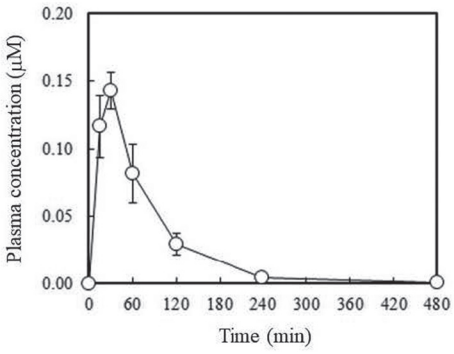

Fig. 3. Plasma Concentration Profile of HBA after the Oral Administration of Purified HBA

Values are the means ± SEM of three rats.

In the ternatin absorption study,[7] we classified the ternatin, malonylated delphinidin 3,3′,5′-triglucoside, with 3′ and 5′ side chains containing replacing glucosyl (G) and *p*-coumaroyl (C) units, according to the "symmetry" of the substitution patterns of the 3′ and 5′ side chains of delphinidin. We defined ternatins as containing both side chains with no differences, –C, or –G differences as symmetrical type, and ternatin containing both side chains with a difference more than a –CG unit as asymmetrical type. The symmetrical ternatins carrying –G terminals on both side chains (A series) had specifically higher AUC_{0-8}/D values than those of symmetrical ternatins with –C and –G terminals on both side chains (B series): A1(–CGCG, –CGCG) > B1(–CGCG, –CGC), and A3 (–CG, –CG) > B4(–CG, –C),[7] where the entries inside the parentheses indicate the side chain residues on the 3′ and 5′-glucoses of delphinidin. In contrast, the AUC_{0-8}/D of asymmetrical ternatins were similar and independent of their terminal patterns: A2(–CGCG, –CG) = B3(–CGCG, –C) = D2(–CGC, –C).[7]

According to the "symmetry" rules of polyacylated anthocyanins with two side chains attached defined previously,[7] HBA (–CafGCafG, –CafG) with –G terminals attached on both branched chains at the 6″ and 6‴ -glucosyl positions of a sophorosyl moiety was classified as asymmetrical type with both –G terminals such as ternatin A2 (–CGCG, –CG) attached. In addition, the AUC_{0-8}/D value of HBA (409.51 ± 66.03 μM·min/mmol/kg) was similar to that of asymmetrical ternatin A2 (360.88 ± 36.69 μM·min/mmol/kg).[7] Thus, the result of HBA absorption obtained here was in accord with the "symmetry" rules defined in the study of ternatins absorption.[7]

The oral administration of Pn3CafSop5G was not examined in the present study. When the results of anthocyanin absorption using purified cyanidin 3-*O*-β-D-glucopyranoside (Cy3G) from different studies were compared,[10,11] both C_{max}/D and AUC_{0-8}/D values showed the similar levels (0.82 μM/mmol/kg[10] and 1.05 μM/mmol/kg[11] for C_{max}/D, and 194.23 μM·min/mmol/kg[10] and 113.25 μM·min/mmol/kg[11] for AUC_{0-8}/D), although, the experimental designs were completely different. This indicates that results of anthocyanin absorption obtained from different laboratories could be enough compared. Therefore, plasma parameters of Pn3CafSop5G were cited from a previous report of Pn3CafSop5G absorption.[5] The C_{max} and AUC_{0-8} values of Pn3CafSop5G were 0.05 μM and 2.814 μM·min, when 0.0389 mmol/kg of Pn3CafSop5G was

administered orally.[5] The calculated C_{max}/D and AUC_{0-8}/D values of Pn3CafSop5G were 1.285 μM/mmol/kg and 72.34 μM·min/mmol/kg, respectively. Despite its high molecular weight, HBA had an approximately 2-fold higher C_{max}/D value (2.51 ± 0.23 μM/mmol/kg) compared with Pn3CafSop5G. Differences in the AUC_{0-8}/D values between HBA (409.51 ± 66.03 μM·min/mmol/kg) and Pn3CafSop5G were more marked and the AUC_{0-8}/D of HBA was approximately 6-fold higher than that of Pn3CafSop5G. The present results strongly suggest that molecular weight is not the sole factor determining the bioavailability of acylated anthocyanins, as we described previously.[6,7]

When we focused on the structure of Pn3CafSop5G with a caffeoyl (Caf) terminal attached to a side chain at the 6″ position of the sophorosyl moiety, Pn3CafSop5G (–Caf, –H) was defined as asymmetrical type, similar to HBA with asymmetrical branched chains (–CafGCafG, –CafG) as discussed above. The present results clearly demonstrated that –G terminals on the branched chains on asymmetrical HBA improved its AUC_{0-8}/D, despite its large molecular weight compared with Pn3CafSop5G with a –Caf terminal attached to 6″-position, in contrast to the bioavailability of asymmetrical ternatins reported previously.[7] Taken together, the relatively high absorption of polyacylated anthocyanins with –G terminals attached to both chains is not related on the symmetry of the acylated sugar chains.

Ternatins attach side chains comprised of G and C moieties to the 3′ and 5′ positions of delphinidin, whereas HBA attaches the branched chains comprised of G and Caf moieties to the 6″ and 6‴ positions of the sophorosyl moiety at the 3 position of peonidin. These structural differences, including aglycone, aromatic ring residues, substitution position, and symmetries of acylated sugar chains, as well as various combinations of these, might modulate the bioavailability of polyacylated anthocyanins, further complicating this system. Further studies on the absorption of polyacylated anthocyanins with two acylated sugar chains attached with different substitution positions such as platyconin (substituted at the 3 and 7 positions of delphinidin) and gentiodelphin (substituted at the 3′ and 5 positions of delphinidin) are required to clarify this point.

It was reported that a typical non-acylate anthocyanin, Cy3G, is transported by intestinal glucose transporter 2 (GULT-2) in Caco-2 cells[12] and organic anion transport polypeptide (OATP) in rat intestinal membrane experiments.[13] In contrast, cyanidin 3-*O*-(6-*O*-(6-*O*-feruloyl-β-D-glucopyranosyl)-(2-*O*-β-D-xylopyranosyl))-β-D-galactopyranoside derived from purple carrot was transported via OATP alone and GULT-2 mediated transport was not involved.[13] The terminal feruloyl moiety of arabinoxylan oligosaccharide inhibits its GLUT-2 mediated transport in Caco-2 cells,[14] although, inhibition of GULT-2 by the inside feruloyl moieties on the acyl sugar chain was not clarified. Similarly, terminal acyl moieties, including –C, –Caf, and –feruloyl residues, attached to the acylated sugar chains of acylated anthocyanins may inhibit the intestinal GLUT-2 mediated transport of acylated anthocyanins. Therefore, acylated anthocyanins carrying –G terminals on both acylated sugar chains, may not inhibit GULT-2 transport, and are possibly absorbed via OATP and GLUT-2 similar to Cy3G, leading to their higher absorption compared with acylated anthocyanins containing –C or –Caf terminals, which are transported by OATP alone.

In conclusion, this is the first study to show that HBA is directly absorbed from the gastrointestinal tract in its original polyacylated forms to the same extent as ternatins. Asymmetrical HBA with –G terminals on both side chains showed a higher absorption, despite its large molecular weight, compared with asymmetrical Pn3CafSop5G with –Caf terminals on 6″-position. Studies on the absorption mechanism of polyacylated anthocyanins from the gastrointestinal tract via various transporters such as OATP and GLUT-2 are now in progress.

Conflict of interest The authors declare no conflict of interest.

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