α-Glucosidase Inhibitory Action of Natural Acylated Anthocyanins. 2. α-Glucosidase Inhibition by Isolated Acylated Anthocyanins

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Four diacylated pelargonidin (Pg: SOA-4 and SOA-6), cyanidin (Cy: YGM-3), and peonidin (Pn: YGM-6) 3-sophoroside-5-glucosides isolated from the red flowers of the morning glory, *Pharbitis nil* cv. Scarlett O'Hara (SOA), and the storage roots of purple sweet potato, *Ipomoea batatas* cv. Ayamurasaki (YGM), were subjected to an α-glucosidase (AGH) inhibitory assay, in which the assay was performed with the immobilized AGH (iAGH) system to mimic the membrane-bound AGH at the small intestine. As a result, the acylated anthocyanins showed strong maltase inhibitory activities with IC_{50} values of <200 μM, whereas no sucrase inhibition was observed. Of these, SOA-4 [Pg 3-*O*-(2-*O*-(6-*O*-(*E*-3-*O*-(β-D-glucopyranosyl)caffeyl)-β-D-glucopyranosyl)-6-*O*-*E*-caffeyl-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside] possessed the most potent maltase inhibitory activity (IC_{50} = 60 μM). As a result of a marked reduction of iAGH inhibitory activity by deacylating the anthocyanins, that is, Pg (or Cy or Pn) sophoroside-5-glucoside, acylation of anthocyanin with caffeic (Caf) or ferulic (Fer) acid was found to be important in the expression of iAGH (maltase) inhibition. In addition, the result that Pg-based anthocyanins showed the most potent maltase inhibition, with an IC_{50} value of 4.6 mM, and the effect being in the descending order of potency of Pg > Pn ≒ Cy strongly suggested that no replacement at the 3′(5′)-position of the aglycon B-ring may be essential for inhibiting iAGH action.

Keywords: *α-Glucosidase; acylated anthocyanins; inhibition; non-insulin-dependent diabetes mellitus*

INTRODUCTION

Recent studies on polyphenols have demonstrated that they possess diverse pathophysiological properties such as antioxidant and antihypertensive activities (*1*, *2*). Honda and Hara examined α-glucosidase (AGH, EC 3.2.1.20) inhibition of polyphenols regarding catechins and theaflavins, in which esterification of polyphenols was responsible for the increase in inhibition power (*3*). In addition, anthocyanins showed inhibition of lipid oxidation as well as catechins (*4*). As expected, these findings have led us to a further functional investigation of anthocyanins from the point of view of their physiological activity. On the basis of our previous finding (*5*) that the anthocyanin extracts from various plants showed an in vitro suppression effect of postprandial glucose absorption, an alternative function of anthocyanins might be the retardation of AGH action.

Hence, in this study we have investigated four diacylated and three deacylated anthocyanidin 3-sophoroside-5-glucosides having different aglycons of pelargonidin (Pg), cyanidin (Cy), and peonidin (Pn) isolated from the red flowers of the morning glory, *Pharbitis nil* cv. Scarlett O'Hara (SOA), and the storage roots of the purple sweet potato, *Ipomoea batatas* cv. Ayamurasaki (YGM), to clarify their structural effect on AGH inhibitory action. Additional study was done to investigate the effect of flavylium cation structure of anthocyanin on the inhibition of AGH activity at the intestinal condition (pH 6.8).

MATERIALS AND METHODS

Materials. α-Glucosidase (EC 3.2.1.20) from rat intestinal acetone powder was purchased from Sigma Chemical Co. (St. Louis, MO). Papain (14 units/mg of solid, from papaya latex, EC 3.4.22.2) was the product of Nacalai Tesque (Kyoto, Japan). CNBr-activated Sepharose 4B was purchased from Pharmacia Biotech AB (Uppsala, Sweden). Two plant materials, that is, the red flowers of SOA and the storage roots of YGM, were used in this study. SOA were grown on a farm of Minami-Kyushu University, and YGM storage roots were obtained from Kyushu National Agricultural Experiment Station. SOA flowers were dried at 45 °C overnight and stored to dryness until use for extraction. Other reagents were of analytical grade and used without further purification.

Preparation of Anthocyanins from Plants. Dried flower petals (25 g) of SOA were immersed in 1 L of 5% acetic acid (AcOH) solution overnight, and then the extract was applied on an Amberlite XAD-2000 resin column (∅ 45 × 300 mm, Rohm and Haas) with the elution of 5% AcOH in 70% EtOH. After the evaporation, the residue was dissolved in 50% AcOH in MeOH, followed by centrifugation. The supernatant was

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10.1021/jf0012502 CCC: $20.00
Published on Web 03/07/2001

precipitated with excess diethyl ether, and the precipitate was collected to dryness in vacuo, to obtain SOA crude extract as a red powder. The SOA extract was dissolved in 0.1% trifluoroacetic acid (TFA)/EtOH = 6:4, v/v, and chromatographed on a PVP column (∅ 45 × 300 mm, polyclar AT, GAF Chemicals Co.) with the same solvent. From the pigment fractions, SOA-4 and -6 were isolated by application on a preparative ODS-HPLC (L-6200 intelligent pump system, Hitachi Co.) on a column (Inertsil ODS 5, ∅ 20 × 250 mm, GL Sciences Inc.) with the isocratic solvent system of A (15% AcOH)/B (15% AcOH, 30% CH_3CN) = 70:30 to 50:50, v/v, at 520 nm according to the procedure given in ref *6*. Each isolated fraction was evaporated to dryness, dissolved in a minimum amount of TFA, and precipitated with the addition of excess diethyl ether; the precipitate was collected and dried in vacuo. SOA-4 and -6 were obtaind as red powders of TFA salt. YGM-3 and -6 were isolated as TFA salts from YGM storage roots according to a method given in a previous paper (*7*). From the acylated anthocyanins, three deacylated anthocyanins, Pg, Cy, and Pn 3-*O*-(2-*O*-(-*β*-D-glucopyranosyl)-*β*-D-glucopyranoside)-5-*O*-*β*-D-glucopyranosides (3-sophoroside-5-glucosides or 3S5G for short), were prepared by alkaline deacylation reported elsewhere (*8*). The anthocyanidin 3-sophoroside-5-glucosides were confirmed and cochromatographed by HPLC with corresponding authentic pigments (*9*−*11*). Analytical ODS-HPLC (L-6200 intelligent pump system, Hitachi) was run on a column (Inertsil ODS-3, ∅ 4.6 × 250 mm, GL Sciences Inc.) with the liner gradient elution system of A (1.5% H_3PO_4)/B (1.5% H_3PO_4, 20% AcOH, 25% CH_3CN) = 25:75 to 55:45, v/v, for 60 min at 520 or 310 nm.

Structural Analysis of SOA-4 and -6. Acid and alkaline hydrolyses and H_2O_2 oxidation of isolated SOA-4 and -6 were performed according to previous methods (*8*). UV−vis spectra were recorded on a V-550 (JASCO Co., Japan) spectrophotometer in 0.01% HCl/MeOH. FABMS spectra were recorded on a JMS SX-102 (JEOL) in MeOH with the Magic Bullet (a dithioerythritol/dithiothreitol mixture, $C_4H_{10}O_2S_2$ = 154) as a matrix and measured in a positive mode. ESI-TOFMS spectra were measured on a Mariner Biospectrometry workstation (PerSeptive Biosystems Inc.) in 1% AcOH/50% CH_3CN in a positive mode. ^1H and ^{13}C NMR spectra were run on an Alpha-400 (JEOL) in DMSO-d_6/CF_3COOD = 9:1 with TMS as the internal standard. SOA-4 was cochromatographed to identify with an authentic anthocyanin, which was isolated from the same plant and it structure determined by Lu et al. (*9*).

Analytical Data of SOA-4. UV−vis λ_{max} (0.01% HCl/MeOH) 510 (not shifted bathochromically with $AlCl_3$), 288, 325 nm; $E_{440}/E_{vis} = E_{440}/E_{510}$ = 22%, $E_{UV}/E_{vis} = E_{288}/E_{510}$ = 105%, E_{325}/E_{510} = 108%; ESI-TOFMS, *m*/*z* 1243 ($M = C_{79}H_{91}O_{45})^+$.

Analytical Data of SOA-6. UV−vis λ_{max} (0.01% HCl/MeOH) 510 (not shifted bathochromically with $AlCl_3$), 288, 331 nm; $E_{440}/E_{vis} = E_{440}/E_{510}$ = 22%, $E_{UV}/E_{vis} = E_{288}/E_{510}$ = 95%, E_{331}/E_{510} = 98%; FABMS, *m*/*z* 1081 ($M = C_{79}H_{91}O_{45})^+$; ^1H NMR *δ* 8.92 (Pg-4, s), 6.98 (Pg-6, s), 8.58 (Pg-2′, d, *J* = 8.3 Hz), 7.10 (Pg-3′, d, 8.3), 7.10 (Pg-5′, d, 8.3), 8.58 (Pg-6′, d, 8.3), 4.85 (Gb-1, d, 7.3), 3.17 (Gb-2, t, 8.0), 3.30 (Gb-3, m), 3.30 (Gb-4, m), 3.30 (Gb-5, m), 4.09 (Gb-6a, m), 4.09 (Gb-6b, m), 5.13 (Gc-1, d, 7.3), 3.57 (Gc-2, t, 8.3), 3.43 (Gc-3, t, 9.0), 3.30 (Gc-4, m), 3.17 (Gc-5, m), 3.57 (Gc-6a, m), 3.82 (Gc-6b, m), 6.97 (Ga-Caf-2, s), 6.77 (Ga-Caf-5, d, 9.0), 6.86 (Ga-Caf-6, br d, 9.0), 6.08 (Ga-Caf-α, d, 16.0), 6.15 (Ga-Caf-*β*, d, 16.0), 6.90 (Gb-Caf-2, s), 6.75 (Ga-Caf-5, d, 8.8), 6.80 (Gb-Caf-6, br d, 9.0), 5.85 (Gb-Caf-α, d, 16.0), 5.95 (Gb-Caf-*β*, d, 16.0);^{13}C NMR *δ* 165.5 (Pg-2), 144.1 (Pg-3), 135.3 (Pg-4), 162.9 (Pg-5), 104.9 (Pg-6), 168.3 (Pg-7), 96.4 (Pg-8), 112.0 (Pg-9), 155.6 (Pg-10), 119.4 (Pg-1′), 119.2 (Pg-2′), 145.7 (Pg-3′), 155.4 (Pg-4′), 117.2 (Pg-5′), 135.2 (Pg-6′), 100.2 (Ga-1), 81.0 (Ga-2), 76.3 (Ga-3), 69.7 (Ga-4), 74.5 (Ga-5), 63.2 (Ga-6), 104.2 (Gb-1), 74.6 (Gb-2), 76.6 (Gb-3), 69.8 (Gb-4), 74.3 (Gb-5), 63.0 (Gb-6), 102.0 (Gc-1), 73.3 (Gc-2), 77.2 (Gc-3), 70.1 (Gc-4), 77.8 (Gc-5), 61.0 (Gc-6), 125.6 (Ga-6-Caf-1), 115.3 (Ga-6-Caf-2), 145.7 (Ga-6-Caf-3), 148.5 (Ga-6-Caf-4), 116.0 (Ga-6-Caf-5), 121.5 (Ga-6-Caf-6), 113.7 (Ga-6-Caf-α), 145.5 (Ga-6-Caf-*β*), 166.7 (Ga-6-Caf-carbonyl), 125.6 (Gb-6-Caf-1), 115.1 (Gb-6-Caf-2), 145.2 (Gb-6-Caf-3), 148.5 (Gb-6-Caf-4), 116.0 (Gb-6-Caf-5), 121.3 (Gb-6-Caf-6), 113.8 (Gb-6-Caf-α), 144.4 (Gb-6-Caf-*β*), 166.5 (Gb-6-Caf-carbonyl).

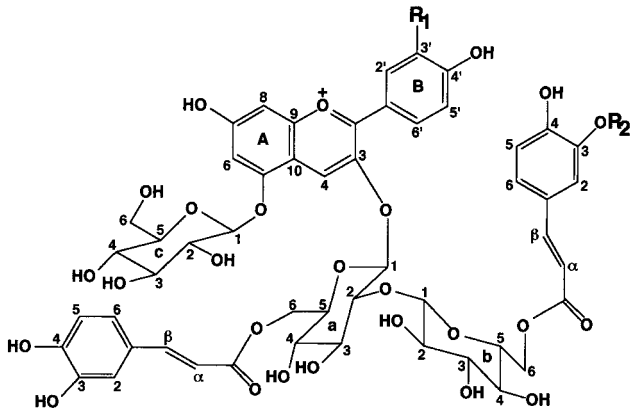
Immobilized AGH (iAGH) Assay System. The immobilization of AGH partially purified from rat acetone powder onto CNBr-activated Sepharose 4B and the iAGH assay system were the same as reported in our previous paper (*12*). The liberated glucose was assayed by HPLC (Shimadzu LC-10A). The HPLC conditions were as follows: column, $5NH_2$-MS (∅ 4.6 × 250 mm, Nacalai Tesque); solvent, 75% CH_3CN (1.0 mL/min); RI detection (RI-300, JASCO, Tokyo, Japan). The concentration of AGH inhibitor required to inhibit 50% of the AGH activity under the assay conditions was defined as the IC_{50} value.

The effect of pH on the iAGH inhibitory activity of anthocyanins was determined as follows: 1.0 mg of anthocyanin (SOA-4) dissolved in 1.0 mL of intestinal fluid solution [0.1 M phosphate buffer (pH 6.8)] described in the *Japanese Pharmacopoeia* (JP XIII) was incubated at 37 °C at different fixed incubation times up to 24 h. At each fixed incubation time, the anthocyanin solution was subjected to the iAGH assay system for AGH inhibitory activity and UV−vis spectrophotometry (Shimadzu UV-300).

RESULTS AND DISCUSSION

Isolation of Anthocyanins. In our preliminary screening experiments for iAGH inhibitors from natural resources (*5*), the aqueous AcOH soluble fractions of SOA (IC_{50} = 0.167 mg/mL against maltase) and YGM (IC_{50} = 0.261 mg/mL against maltase) plants showed high iAGH inhibitory activity as potent as that of green tea extract (IC_{50} = 0.215 mg/mL against maltase) with in vivo activity. To identify any natural inhibitors present in them, isolation by preparative ODS-HPLC was done for each fraction. As a result of NMR and FABMS analyses, the main isolates of YGM extract were diacylated anthocyanins, YGM-3 and YGM-6 [Cy and Pn 3-*O*-(2-*O*-(6-*O*-*E*-Fer-*β*-D-glucopyranosyl)-6-*O*-*E*-Caf-*β*-D-glucopyranoside)-5-*O*-*β*-D-glucopyranosides, respectively], which had been described previously (*7*). As for the SOA extract, diacylated anthocyanins SOA-4 and -6 were isolated as well. Their structures were determined by chemical and spectral analyses. The SOA-6 structure was confirmed by ^1H and ^{13}C NMR techniques incorporating double-quantum filtered correlation spectroscopy (DQF-COSY), heteronuclear multiple-quantum coherence (HMQC), heteronuclear multiple-bond correlation (HMBC), and nuclear Overhauser effect difference spectroscopy (NOEDF). In the low magnetic field, the ^1H NMR spectrum of SOA-6 shows the presence of a Pg moiety based on proton signals on the benzopyrylium nucleus and 1,4-disubstituted aromatic B-ring. The presence of a trans (*E*)-Caf group is confirmed with the l,2,4-disubstituted benzene and the (*E*)-olefinic proton signals with large coupling constant ($J_{\alpha,\beta}$ = 16.0 Hz). In the high magnetic field, the spectra also show all sugars to be of *β*-D-glucopyranosyl configuration because of the resonances at lower magnetic field (δ_H 4.85−5.62) of all anomeric protons and the large *J* values (*J* = 7.3−9.0 Hz) of the anomeric and the ring protons. Because glucose b (G_b) links on G_a-2OH due to the glycosylation shift of G_a-2H (at δ_H 4.01) and G_a-2C (at δ_C 81.0), the interglycosidic linkage is *β*-D-G_b (1→2) G_a, a sophoroside.

The relationships among an aglycon, three sugars, and two acyl groups were confirmed by NOEDS and HMBC measurements. In the NOEDS spectra, three informative NOE signals between Pg-4H and G_a-1H (Pg-4H/G_a-1H), G_a-2H/G_b-1H, and Pg-6H/G_c-1H indicated that G_a, G_b, and G_c connected at Pg 3-OH, at G_a-2OH, and at Pg 5-OH through a glycosyl bond, respectively.



	R_1	R_2
SOA-4	H	Glc
SOA-6	H	H
YGM-3	OH	Me
YGM-6	OMe	Me

Figure 1. Structures of anthocyanins used in this experiment. Glc, glucose.

In the 2D-HMBC spectra, the clear $^1H-^{13}C$ cross-peaks between G_a-1H and Pg-3-carbon signals (G_a-1H/ Pg-3C), G_b-1H/ G_a-2C, G_a-2H/ G_b-1C, and G_c-1H/ Pg-5C verified the connections G_a/Pg 3-OH, G_b/G_a-2OH, and G_c/Pg 5-OH, respectively. The 6-acylation was also supported by deshielding shifts of G_a-6Hs (δ_H 4.32, 4.43) and G_a-6C ($\Delta\delta_C$ 63.2), and G_b-6Hs (δ_H 4.09) and G_b-6C ($\Delta\delta_C$ 63.0) clearly indicated each caffeic acid linked to G_a- and G_b-6OH, respectively. Moreover, the ^1H NMR assignment data of SOA-6 were almost the same as those of the corresponding anthocyanin reported by Saito et al. (*13*).

Thus, the structures of SOA-4 and -6 were respectively determined as Pg 3-*O*-(2-*O*-(6-*O*-(*E*-3-*O*-(*β*-D-glucopyranosyl) Caf)-*β*-D-glucopyranosyl)-6-*O*-*E*-Caf-*β*-D-glucopyranoside)-5-*O*-*β*-D-glucopyranoside and Pg 3-*O*-(2-*O*-(6-*O*-*E*-*O*-Caf-*β*-D-glucopyranosyl)-6-*O*-*E*-Caf-*β*-D-glucopyranoside)-5-*O*-*β*-D-glucopyranoside, which had already been established by Lu et al. (*9*) and Saito et al. (*13*), respectively. These four anthocyanins had a common diacylated anthocyanidin 3-sophoroside-5-glucoside structure as represented in Figure 1.

iAGH Inhibition by SOA and YGM Anthocyanins. Primarily, iAGH inhibition study of these four anthocyanins against maltase were performed. Although data are not shown, no sucrase inhibition was observed for the anthocyanins. As shown in Table 1, iAGH inhibitory activities of all the isolated anthocyanins were >5 times higher than that of the natural inhibitor, D-xylose (IC_{50} = 1190 μM); among them, SOA-4 possessed the most potent activity as shown by an IC_{50} value of 60 μM, and their inhibitory behavior was in the order of SOA-4 > SOA-6 > YGM-3 ≒ YGM-6. Asano et al. reported that oral administration of D-xylose for Wistar rats and human volunteers was confirmed to suppress the 30-min postprandial blood glucose level (PBG) (ΔPBG > 20 mg/dL) at the dose of 0.1 g/kg and 7.5 g/person, respectively (*14*). Judging from the results of iAGH inhibitory activities of the anthocyanins, therefore, $<^1/_5$-fold lower dose of them than D-xylose would be enough to achieve the equivalent PBG lowering effect of D-xylose in vivo. On the other hand, these four anthocyanins were found to be poor natural iAGH inhibitors, compared with the therapeutic AGH inhibitors voglibose (IC_{50} = 0.0055 μM) and acarbose (IC_{50} = 0.426 μM). From the viewpoint of their use as physiologically functional food material, their weaker activities must be, however, acceptable for their daily intake to make a moderate PBG control.

Table 1. iAGH[a] (Maltase) Inhibitory Activities of Isolated Acylated Anthocyanins, D-Xylose, and the Therapeutic Drugs Voglibose and Acarbose

	IC_{50} value	
compound[b]	μM	mg/mL
SOA-4	60	0.081
SOA-6	107	0.128
YGM-3	193	0.239
YGM-6	200	0.245
D-xylose	1190	
voglibose	0.0055	
acarbose	0.426	

[a] AGH inhibitory assay was performed on the immobilized assay system detailed in the text by using 10 mM maltose solution. [b] The abbreviated names of anthocyanins refer to Figure 1.

The previous paper (*5*) revealed that the SOA and YGM extracts inhibited the iAGH (maltase) action with IC_{50} values of 0.167 and 0.261 mg/mL, respectively. Considering the iAGH inhibition powers of SOA-4 (IC_{50} = 0.081 mg/mL) and SOA-6 (IC_{50} = 0.128 mg/mL) shown in Table 1, the two SOA anthocyanins were found to be prominent iAGH inhibitors in the SOA extract. YGM-3 (IC_{50} = 0.239 mg/mL) and YGM-6 (IC_{50} = 0.245 mg/mL) anthocyanins were the main contributing inhibitors present in the YGM extract (IC_{50} = 0.261 mg/mL) as well. Thus, no iAGH inhibitors other than the anthocyanins were present in their extracts.

Anthocyanin Structure—iAGH Inhibitory Activity Relationship. To confirm the structural factors being attributed to powerful iAGH inhibition of SOA, and YGM anthocyanins, their deacylated anthocyanins were subjected to the iAGH assay. As summarized in Table 2, three deacylated anthocyanins with different anthocyanidins, that is, Pg (or Cy or Pn) 3S5G, were examined in this experiment. As a result, the iAGH inhibitory activities of these deacylated anthocyanins

Table 2. iAGH (Maltase) Inhibitory Activities of Deacylated Anthocyanins

anthocyanin[a]	IC_{50} value (μM)
Pg3S5G	4610
Pn3S5G	14100
Cy3S5G	18200

[a] Abbreviations: Pg, pelargonidin; Pn, peonidin; Cy, cyanidin; 3S5G, 3-*O*-(2-*O*-β-D-glucopyranosyl)-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside.

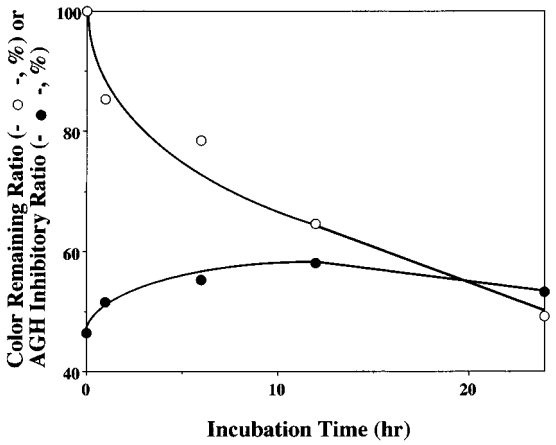


Figure 2. Effect of incubation time at pH 6.8 for up to 24 h on AGH inhibitory activity of SOA-4. SOA-4 (0.1 mg/mL) was incubated at 37 °C and the AGH inhibition assayed (%, ●). The ratio (%, ○) of E_{537} at a definite incubation time against that at 0 h was used as an index of decoloration of SOA-4.

markedly decreased by a factor of $^1/_{70}-^1/_{90}$ compared to their acylated ones. This indicated that, in any case, acylation of anthocyanin with Caf or Fer must be important in the expression of iAGH (maltase) inhibition. For sucrase inhibition study by tea polyphenols, esterified catechins such as epicatechin gallate were reported to be stronger inhibitors than nonesterified ones (*3*). Similar AGH inhibitory expression by acylation was observed in sulfoquinovosyldiacylglycerol from edible brown algae (*15*). Therefore, the more potent iAGH inhibition by acylated SOA and YGM anthocyanins compared with that of the corresponding deacylated ones would be due to higher affinity with the enzyme active site. In addition, useful structural information on iAGH inhibitory expression by acylated anthocyanins was obtained from the inhibition study by deacylated anthocyanins. Among the three deacylated anthocyanins, Pg-based anthocyanin (Pg3S5G) showed the most potent maltase inhibition with an IC_{50} value of 4.6 mM, and the effect was in the descending order of potency of Pg3S5G > Cy3S5G ≒ Pn3S5G (Table 2). Interestingly, the order was almost same in the case of acylated anthocyanins [SOA-4 (Pg type) > SOA-6 (Pg type) > YGM-3(Cy type) ≒ YGM-6 (Pn type)] as shown in Table 1. Therefore, these findings strongly suggested that no replacement at the 3′(5′)-position of the B-ring may be essential for inhibiting iAGH action.

Effect of pH on the iAGH Activity of Acylated Anthocyanins. Because anthocyanins are liable to decoloration with increasing pH (*16*), it is important to investigate whether the isolated anthocyanins show long-term iAGH inhibition power in small intestinal fluid as much as in the definite assay condition (<60-min incubation). It is well-known that anthocyanin as a flavylium cation form has a stable colored form only at lower pH of the solution, whereas with increasing pH its transformation to the equilibrium mixture of the colorless carbinol pseudobase and the retrochalcone proceeds via the rapid generation of quinoidal anhydrobase (*17*). Figure 2 shows the AGH inhibitory change of SOA-4 (0.1 mg/mL) during the incubation at 37 °C for 24 h at pH 6.8. Interestingly, the inhibitory activity increased as incubation or its decoloration reaction proceeded up to 24 h. This finding suggested that molecular species other than flavylium cation might contribute to the whole AGH inhibition power. The acylated side-chain structure of the anthocyanin would be, in turn, important for enhancing the AGH inhibition. The role of acylated side structure of anthocyanins in inhibiting iAGH action is now under investigation.

ABBREVIATIONS USED

AGH, α-glucosidase; Pg, pelargonidin; Cy, cyanidin; Pn, peonidin.

ACKNOWLEDGMENT

We are indebted to Dr. O. Yamakawa, Kyushu National Agricultural Experiment Station, for donating the storage roots of purple sweet and we thank Dr. N. Saito, Meiji-Gakuin University, for his gift of the authentic anthocyanins from SOA flowers.

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Received for review October 18, 2000. Revised manuscript received January 16, 2001. Accepted January 16, 2001. Part of this work was supported by a Grand-in Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, and Culture of Japan (No. 11794007).

JF0012502