α-Glucosidase Inhibitory Action of Natural Acylated Anthocyanins. 1. Survey of Natural Pigments with Potent Inhibitory Activity

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α-Glucosidase (AGH) inhibitory study by natural anthocyanin extracts was done. As the result of a free AGH assay system, 12 anthocyanin extracts were found to have a potent AGH inhibitory activity; in particular, *Pharbitis nil* (SOA) extract showed the strongest maltase inhibitory activity, with an IC_{50} value of 0.35 mg/mL, as great as that of *Ipomoea batatas* (YGM) extract (IC_{50} = 0.36 mg/mL). Interestingly, neither extract inhibited the sucrase activity at all. For the immobilized assay system, which may reflect the pharmacokinetics of AGH at the small intestine, SOA and YGM extracts gave more potent maltase inhibitory activities than those of the free AGH assay, with IC_{50} values of 0.17 and 0.26 mg/mL, respectively. Both extracts also inhibited α-amylase action, indicating that anthocyanins would have a potential function to suppress the increase in postprandial glucose level from starch.

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

INTRODUCTION

Non-insulin-dependent diabetes mellitus (NIDDM), which is one of the main adult diseases, is caused by the secretory decrease in insulin from pancreatic Langerhans β cell or the lowering of insulin resistance due to excess glucose absorption (*1*). Serious side effects such as retinopathy, neuropathy, and cataracts (*2*) are also brought about by its long-term manifestation. At present, the direct clinical therapy and prophylaxis in NIDDM is to optimize or control the postprandial blood glucose (PBG) level (*3*).

α-Glucosidase (AGH, EC 3.2.1.20), which is a membrane-bound enzyme located at the epithelium of the small intestine, catalyzes the cleavage of glucose from disaccharides (*4*). Thus, the retardation of the action of AGH by any inhibitors may be one of the most effective approaches to control NIDDM (*5*). In Japan, acarbose and voglibose as therapeutic α-glucosidase inhibitors are widely used to delay glucose absorption from the small intestine (*6*, *7*). Long-term treatment with acarbose (300 mg/day) has demonstrated therapeutic efficacy resulting in a significant decrease of 1-h PBG levels after 3 months of the protocol in diabetic subjects with few side effects such as flatulence and diarrhoea (*6*).

From this point of view, we have investigated AGH inhibitors from natural resources to develop a physiologically functional food, for which *Bacillus licheniformis* alkaline protease hydrolysate derived from sardine muscle (IC_{50} = 48.7 mg/mL) (*8*) and the isolated peptides (IC_{50} Tyr-Tyr-Pro-Leu = 3.7 mM; IC_{50} Val-Trp = 22.6 mM) (*9*) fulfilled our demand. Many researchers have also investigated natural products with AGH inhibitory activity as well. Recently, Shi et al. determined that the extracts from *Sophora* plants dose-dependently suppressed the increase in 30-min PBG levels of a rat given sucrose and had more potent AGH (sucrase) inhibitory activity (IC_{50} = 19 μg/mL) than catechin (IC_{50} = 291 μg/mL) (*10*). These results permit us to investigate unknown functions of flavonoids.

In a series of our studies regarding the isolation of natural pigments, 13 acylated anthocyanins from *Clitoria ternatea* flowers have been identified (*11*, *12*) and 6 acylated anthocyanins from storage roots of purple sweet potato, *Ipomoea batatas*, have been identified (*13*). The anthocyanins commonly possessing 2-phenylbenzophyrylium or flavylium structure have been found to have strong antioxidant activity as well other flavonoids (*14*, *15*), whereas no attention had been directed to AGH inhibition by anthocyanins. Hence, in the present paper, many anthocyanin extracts from various plants were primarily subjected to AGH inhibition study. An AGH inhibition study or survey was made by our proposed immobilized rat intestinal AGH assay system with comparison of the conventional free AGH assay one; the proposed assay may reflect pharmacokinetics of AGH in the small intestine.

MATERIALS AND METHODS

Materials. α-Glucosidase (EC 3.2.1.20) from rat intestinal acetone powder and human saliva α-amylase (EC 3.2. 1.1) were purchased from Sigma Chemical Co. (St. Louis, MO). *p*-Nitrophenyl α-D-glucopyranoside (PNP-G) as a synthetic substrate was obtained from E. Merck (Darmstadt, Germany).

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10.1021/jf001251u CCC: $20.00
Published on Web 03/06/2001

Table 1. Anthocyanin Extracts from Various Plants Used in This Study

scientific name	English name	abbreviation
Clitoria ternatea L. flower	butterfly pea	ternatin
Ipomoea batatas L. root	sweet potato	YGM
Brassica oleracea L. leaf	cabbage	BOC
Raphanus sativus L. root	radish	RPS
Perilla ocimoides L. leaf	perilla	POP
Dioscorea alata L. tuber	yam	DOA
Pisum sativum L. pod	pea	PSP
Sambucus nigra L. berry	elderberry	SNB
Fatsia japonica L. berry skin		FJB
Rubus loganbaccus berry	boysenberry	RLB
Pharbitis nil cv. Scarlett O'Hara flower	morning glory	SOA
Houttuynia cordata Thunb. leaf		HCT
Salvia splendens flower	salvia	SSS
Zea mays L. seed coat	corn	ZML
Vitis vinifera L. berry skin	grape	VG
Vaccinium spp. berry skin	blueberry	VSB

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). Hot-water extract of green tea was prepared according to our previous paper (*8*). Sixteen plant materials shown in Table 1 were used in this study. Almost all plant materials were grown on a farm of Minami-Kyushu University. They 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 Crude Anthocyanin Extracts from Plants. The dried samples (25 g) were immersed in 1 L of 5% acetic acid (AcOH) solution overnight, and then the extract was placed on an Amberlite XAD-2000 resin column (Ø 45 × 300 mm) 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 added with excess diethyl ether, and the precipitate was dried in vacuo.

Assay for Free AGH Inhibitory Activity. The AGH inhibitory activity was assayed according to the procedure described elsewhere (*16*). Briefly, the reaction was started by adding 40 μL of AGH solution to 760 μL of substrate solution (0.1 M phosphate buffer) adjusted to pH 6.8, followed by incubation at 37 °C for 20 min in 0.7 mM PNP-G, 30 min in 6 mM maltose, or 60 min in 45 mM sucrose solution. After 10 min of boiling, the AGH activity in each solution was measured. When PNP-G was used as substrate, the AGH activity was determined by monitoring the *p*-nitrophenol released from PNP-G at 400 nm (Shimadzu UV-1200 spectrophotometer, Kyoto, Japan). When maltose or sucrose was used, the liberated glucose was assayed by HPLC (Shimadzu LC-10A). The HPLC conditions were as follows: column, $5NH_2$-MS (Ø 4.6 × 250 mm, Nacalai Tesque); solvent, 75% CH_3CN (l.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.

Preparation of Immobilized AGH onto CNBr-Activated Sepharose 4B. The immobilization of AGH partially purified from rat acetone powder by papain onto CNBr-activated Sepharose 4B was performed according to our previous report (*17*). At first, the support (50 mg of dry gel) was immersed in 2 mL of 1 mM HCl for 15 min. After a rinse with 1 mM HCl and a coupling buffer (0.1 M borate buffer containing 0.5 M NaCl), 1 mL of the prepared AGH (2 mg) was added to the support. After incubation at 20 °C for 2 h, the support was washed with the coupling buffer, followed by the addition of 1 mL of 0.1 M β-alanine as a blocking reagent. The preparations were kept at 4 °C in a model of intestinal fluid [0.1 M phosphate buffer (pH 6.8)] described in the *Japanese Pharmacopoeia* (JP XIII).

Assay for Immobilized AGH Inhibitory Activity. Immobilized AGH activity was assayed by adding 200 μL of inhibitor solution and 800 μL of 10 mM maltose solution to the immobilized AGH support (10 mg of wet gel). After incubation at 37 °C for 30 min, the inhibitory activity was determined by the difference in the AGH activity or the liberated glucose with or without inhibitor.

Assay for α-Amylase Inhibitory Activity. The assay for the inhibitory activity on human saliva α-amylase was done by using Amylase-Test Wako (Wako Pure Chemical Institute, Tokyo, Japan); the decrease in starch by the action of α-amylase was measured at 660 nm.

RESULTS

Primarily, the free AGH inhibitory activities of 16 plant materials were investigated using PNP-G, by which the overall AGH activity can be evaluated as the sum of maltase and/or sucrase activities. Figure 1 summarizes the inhibitory profiles of all of the anthocyanin extracts (0.5 mg/mL) against the free AGH activity. Here, five water-insoluble extracts (CTUL, SSS, ZMC, VG, and VSB extracts) were dissolved in dimethyl sulfoxide (DMSO), which did not affect the AGH activ-

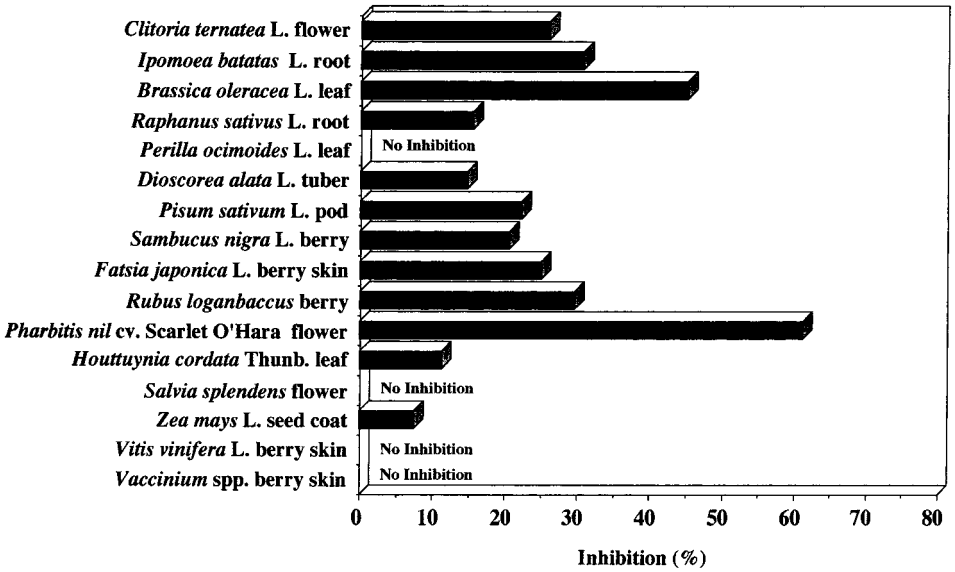

Figure 1. Inhibition profile of various anthocyanin extracts against free AGH. Free AGH inhibition assay was performed by using 0.7 mM PNP-G as a substrate at 37 °C for 20 min. The concentration of the extract was set at 0.5 mg/mL.

Table 2. Free AGH Inhibitory Activity of Anthocyanin Extracts When PNP-G,[a] Maltose, or Sucrose Was Used as a Substrate

	IC_{50} (mg/mL)		
extract[b]	PNP-G	maltose	sucrose
SOA	0.22	0.35	NI[c]
BOC	0.67	2.49	NI
YGM	1.10	0.36	—[d]
ternatin	12.0	>20	—
green tea	0.74	0.23	0.52

[a] PNP-G, *p*-nitrophenyl α-D-glucopyranoside. [b] From the list shown in Table 1. [c] NI, no inhibition. [d] Not measured.

Table 3. AGH (Maltase) Inhibitory Activities of Two Active Anthocyanin Extracts[a] Estimated by Free and Immobilized AGH Assay Systems and Their α-Amylase Inhibition

	IC_{50} (mg/mL)		
extract[b]	free AGH	immobilized AGH	α-amylase
SOA	0.35	0.17	0.43
YGM	0.36	0.26	0.61
green tea	0.23	0.22	—[c]

[a] The concentration of the extracts was set at 0.5 mg/mL. [b] From the list shown in Table 1. [c] Not measured.

ity. As shown in Figure 1, 12 anthocyanin extracts were found to give a significant free AGH inhibitory activity for the first time, whereas no inhibition was observed for 4 extracts (POP, SSS, VG, and VSB). Among the active extracts, BOC and SOA extracts were potent inhibitors with >40% inhibitory ratio against free AGH. Other active extracts moderately inhibited the free AGH action with 10−30% inhibitory ratio.

The effects of anthocyanin extracts on AGH, maltase, and sucrase activities were then investigated (Table 2). Although the inhibitory experiments were done for all extracts, the only extracts with maltase inhibition are shown in the table. As a result of free AGH inhibitory study, SOA extract showed the strongest AGH inhibitory activity with an IC_{50} value of 0.22 mg/mL, and the order of the AGH inhibitory abilities were SOA > BOC > YGM ≫ ternatin. Interestingly, the AGH inhibitory activity of SOA extract was 3.5 times stronger than that of the green tea extract (IC_{50} = 0.74 mg/mL) with in vivo PBG reduction activity, in which the tea catechin suppressed the rise of 30-min PBG and insulin levels in rat plasma dose dependently (<80 mg/rat) (30-min PBG = 10 mg of catechin, 168 mg/dL; control = 215 mg/dL) (*18*, *19*). For maltase inhibition, different inhibition profiles against the overall AGH activity were obtained. Namely, the inhibitory activity of BOC extract against maltase (IC_{50} = 2.49 mg/mL) was 1−3.7-fold lower than the overall AGH inhibitory activity. Among the extracts, SOA (IC_{50} = 0.35 mg/mL) and YGM (IC_{50} = 0.36 mg/mL) were sufficient maltase inhibitors, being comparable to green tea extract (IC_{50} = 0.23 mg/mL). None of the extracts inhibited the sucrase activity at all, whereas green tea extract did inhibit. Therefore, it was confirmed that the potent AGH inhibitory activities induced by both SOA and YGM anthocyanin extracts were mainly responsible for their retardation of maltase action. The free maltase inhibitory activity of the four extracts represented in Table 3 was reassayed with our proposed AGH-immobilized CNBr-activated Sepharose 4B support system. As shown in Figure 2, similar inhibitory behavior of the extracts was obtained in both free and immobilized assay systems (SOA > YGM > BOC > ternatin), whereas all of the extracts at the concentration of 0.5 mg/mL inhibited the action of immobilized AGH activity more potently than that of free one. Among the four extracts, SOA and YGM extracts gave 1.4- and 2-fold higher inhibitory activities for the immobilized system than for free one with IC_{50} values of 0.17 and 0.26 mg/mL, respectively.

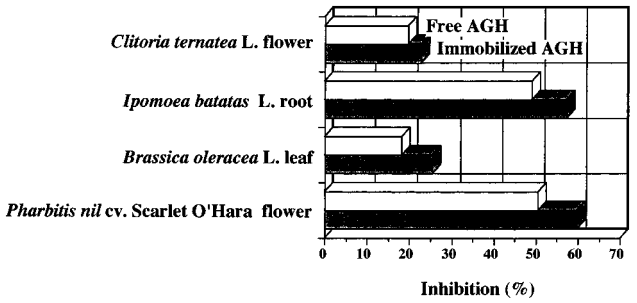

Figure 2. Maltase inhibition profile of four anthocyanin extracts against free and immobilized AGHs. Free and immobilized AGH inhibition assays were performed by using maltose as a substrate at 37 °C. The details for both assays are described in the text. The concentration of the extract was set at 0.5 mg/mL.

Table 3 also presents the α-amylase inhibition by the SOA and YGM extracts. Both the SOA and YGM extracts potently inhibited the α-amylase activity with IC_{50} values of 0.43 and 0.61 mg/mL, respectively, which indicated that the anthocyanin extracts would prominently suppress the intestinal glucose from starch by α-amylase or maltase.

DISCUSSION

To date, there are many studies on the control of the blood glucose level for the prophylaxis of diabetic disease by physiologically functional food materials (*20*−*22*). In this study, we have proved that some anthocyanin extracts from plants possess potent AGH and α-amylase inhibitory activities. Intestinal AGH, which is bound to the brush boarder membrane of the small intestine, is a hydrolase of sucrase−isomaltase complex (*4*), being involved in the terminal digestion of carbohydrates. Thus, it should be noted whether the glucose suppression effect by natural inhibitors is attributed to the retardation of the action of sucrase or maltase. As shown in Table 2, the anthocyanin extracts, in particular, SOA, YGM, and BOC, were found to be definite AGH inhibitors against maltase. No sucrase inhibitory effect was obtained in the extracts. On the other hand, D-xylose was proved to be a sucrase-specific inhibitor. Matsuura and Ichikawa demonstrated the in vivo sucrase inhibitory effect of D-xylose in rats, showing that the 10-min PBG after administration of sucrose (0.35 g/kg, 190 mg/dL) was significantly suppressed by D-xylose feeding (0.12 g/kg, 134 mg/dL) (*23*). Furthermore, Shi et al. reported that water extracts of *Sophora* plants had a 6 times higher inhibitory activity against sucrase than against maltase (*10*). Therefore, anthocyanin extracts showing maltase-specific inhibition in this study had a unique inhibitory action different from that of other natural products. As for α-amylase (Table 3), the extracts were potent inhibitors as well as the tea polyphenols (*24*).

As we have already pointed out (*17*), an AGH inhibitory study should be performed at immobilized state, because AGH is a membrane-bound enzyme, and the resultant AGH inhibitory activity greatly differed from

that in a free AGH system. In the case of voglibose, a therapeutic drug, the AGH immobilization resulted in a 1−9-fold decrease in the IC_{50} value of 62 nM, compared to that of free AGH (IC_{50} = 7.1 nM). In the present study, the AGH (maltase) inhibition of anthocyanin extracts (SOA, YGM, BOC, and ternatin) was enhanced in the immobilized AGH assay system (Figure 2). In particular, SOA and YGM extracts were proved to be powerful AGH inhibitors, and their activities were almost of the same level as that of green tea extract (Table 3).

As is well-known, green tea possesses many polyphenols that were attributed powerful antioxidative effects (*25*). According to the study by Matsumoto et al., tea polyphenols (catechins) were found to have an alternative function, in which they suppressed the rise of 30-min PBG levels at the dose of >10 mg/mL in Wistar rats elevated by sucrose (*19*). Thus, the SOA and YGM anthocyanin extracts with potent AGH inhibitory activity would have a PBG suppression effect as well as tea polyphenols.

In conclusion, this is the first finding that some anthocyanin extracts from plants exert a potent in vitro AGH inhibitory effect contributing to PBG suppression. However, further studies are needed to determine whether the AGH inhibitors present in the extracts are anthocyanins or other compounds.

ABBREVIATIONS USED

AGH, α-glucosidase; PNP-G, *p*-nitrophenyl α-D-glucopyranoside.

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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).

JF001251U