Studies on inhibition of α-amylase and α-glucosidase by components of *Morus australis*

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Contents

Abbreviations

C3G	cyanidin-3-glucoside
C3R	cyanidin-3-rutinoside
1-DNJ	1-deoxynojirimycin
DNS	3,5-dinitrosalicylic acid
GAL-DNJ	2-*O*-α-D-galactopyranosyl deoxynojirimycin
G2-β-PNP	*p*-nitrophenyl-β-D-maltoside
K_i	inhibition constant
LC	liquid chromatography
MRM	multiple reaction monitoring
MS	mass spectrometry
PNPG	*p*-nitrophenyl-α-glucoside
V_{max}	maximum velocity

Chapter 1

General introduction

α-Amylase and α-glucosidase are deeply involved in carbohydrate digestion. Starch comprises a substantial portion of the human diet (1), and is first digested by α-amylase [EC 3.2.1.1]. α-Glucosidase is classified into glycoside hydrolase family GH31 (2), and exists in the human small intestine mucosa responsible for the final step of starch hydrolysis. α-Glucosidase includes maltase [EC 3.2.1.20], glucoamylase [EC 3.2.1.3], sucrase [EC 3.2.1.48], and isomaltase [EC 3.2.1.10]. In mammals, α-glucosidase exists as a maltase-glucoamylase complex and a sucrase-isomaltase complex, which are anchored on small intestinal mucosal brush border (Fig. 1-1). These four α-glucosidase-related enzymes show different α-glycosidic catalytic properties (3), such as hydrolysis of α-1,2-, α-1,4, and α-1,6 bonds. After the digestion by these carbohydrate-digestion enzymes, starch is eventually broken down into glucose and absorbed by human small intestine (4). Using mammalian mucosal α-glucosidases is a better fit to characterize carbohydrate digestion properties, compared to fungal α-glucosidase which is currently applied *in vitro* assays (5).

Diabetes mellitus resulting from deficiency in insulin secretion, can cause a series of complications, such as retinopathy, nephropathy, and neuropathy, when blood sugar rises excessively (6). One of the strategies to treat type-2 diabetes is inhibition of carbohydrate-digestion enzymes. Acarbose, miglitol, and voglibose (Fig. 1-2) are clinically used for this purpose (7). However, patients treated with acarbose experience

side effects such as gas stagnation, flatulence, and diarrhea (8). In order to reduce side effects, natural ingredients in plants with inhibitory effects have received extensive attention.

Various α-glucosidase inhibitors have been identified in plants. They include alkaloids such as vasicine (9), flavonoids such as quercetin (10), polyphenols such as *p*-hydroxycinnamic acid and protocatechuic acid (11), and iminosugars (Fig. 1-2) such as 1-deoxynojirimycin (1-DNJ) (12–16), fagomine (14), and 2-*O*-α-D-galactopyranosyl deoxynojirimycin (GAL-DNJ) (14). Among these ingredients, 1-DNJ strongly inhibits α-glucosidase activity and is anticipated for clinical use (12). 1-DNJ is a glucose analogue with an NH group substituting for the oxygen atom of the pyranose ring (17). Recently, various physiological activities of 1-DNJ have been reported, such as suppression of the elevation of postprandial blood glucose (18), postprandial glycemic control in subjects with impaired glucose metabolism (19), stimulation of adiponectin and GLUT4 expressions adipocytes (20), hypoglycemic effect (21), prevention of diet-induced obesity through increases in adiponectin (22), and suppression of lipid accumulation through activation of the β-oxidation system (23). 1-DNJ, fagomine, and GAL-DNJ can be extracted from mulberry leaves (24). Fagomine and 1-deoxynojirimycin (1-DNJ) can also be found in buckwheat (25). Besides plants, 1-DNJ was also obtained from microorganism source, although information regarding its anti-hyperglycemic effect and safety are limited (26).

In the case of mulberry fruit, anthocyanin and glucose are thought to contribute to the inhibition of α-amylase or α-glucosidase considerably due to the following reasons. Mulberry fruit contains large amounts of anthocyanins (27) and glucose. It was reported that the anthocyanins in mulberry are cyanidin 3-*O*-rutinoside (C3R) (60%) and cyanidin

3-*O*-glucoside (C3G) (38%) (28). Mulberry anthocyanin extract can improve dysfunction in diabetic mice via regulation of AMPK/ACC/mTOR pathway (28), and C3R from baker's yeast inhibited α-glucosidase with IC_{50} value of 19.7 ± 0.24 μM (29). Glucose is reported to be competitive inhibitors on wheat β-amylase (30).

Morus (commonly known as mulberry) is distributed in temperate regions in the world. It is cultivated as a crop for silkworm feed, fruit, and timber. *Morus* contains various α-glucosidase inhibitors, among which 1-DNJ is the most characterized (15–18). *Morus australis*, known as Shimaguwa in Japan, inhabits in Ryukyu islands. It contains more 1-DNJ than *Morus sp.* in Japanese main island (24). Besides iminosugars, *M. australis* contains various phenolic compounds. Guo et al. identified 12 phenolic compounds in *M. australis* root extracts, which were divided into flavonoids, chalcone-derived Diels-Alder adducts, and stilbenes, showing that these compounds exhibited the inhibition activities for α-glucosidase, acetylcholinesterase, tyrosinase (31) and hepatoprotective effects (32). Wang et al. identified 25 phenolic compounds including quercetin, showing that quercetin exhibited the most potent inhibition activity for sucrase, one of α-glucosidase-related enzymes, with IC_{50} values of 8.6 μM (33). Considering that mulberry leaf and fruit also contain other biological components (34), it is a good choice to be able to use mulberry leaf and fruit directly in food.

Consumption of the *M. australis* leaf tea suppressed sucrose-induced increase in blood glucose and insulin levels in human (19, 35). In the inhibition of the hydrolysis of sucrose by sucrase from rat intestine, boiled water extract of powdered *M. australis* leaves exhibited the IC_{50} value of 12 ± 1.3 μg/mL (19). Feeding tests suggested that *M. australis* leaves may exhibit fewer adverse side effects than other α-glucosidase inhibitors (36). In addition, studies indicated that mulberry leaf tea and 1-DNJ diet improved the community

structure of the intestinal flora of rats according to Sheng et al. (37) and Li et al. (38). These results suggest that a high intake of *M. australis* tea might be effective for the prevention of type-2 diabetes. Combining roselle, chrysanthemum, and butterfly pea extracts with mulberry extract showed additive interaction on intestinal maltase inhibition (34). Comparing the inhibitory activities of various components is beneficial to the development of new products.

Based on the assumption that iminosugar, especially 1-DNJ, is a main inhibitory component for α-glucosidase, mulberry leaves and/or fruits are getting attention as a functional substance to suppress postprandial elevation of blood glucose get attention. As for their effects on blood glucose concentration, various studies have been done on mouse or rat (19–23, 25, 26) or human (18, 35). However, limited studies have been reported concerning their enzymatic characterization except for the inhibitory effect of 1-DNJ on maltase and sucrase (19, 36). To pursue the application of mulberry leaves and fruits for this purpose, the followings are thought to be important subjects. First, the concentrations of iminosugar, such as 1-DNJ, fagomine, and GAL-DNJ, in the mulberry leaves and fruits should be accurately quantified. Iminosugars are highly polar and the retention time is very short with a reverse phase column. Hydrophilic interaction chromatography (HILIC) has been reported to be suitable for the separation of iminosugars (24, 39). One of the problems for analysis of iminosugars is that there is no chromophore in iminosugars. As a result, they cannot be analyzed by UV and fluorescence detection. Second, the inhibitory effects of leaf and fruit extracts as well as 1-DNJ, fagomine, and GAL-DNJ on α-amylase and each of the four α-glucosidase-related enzymes, namely maltase, glucoamylase, sucrase, and isomaltase should be characterized. To our knowledge, little is known except for the inhibitory effects of 1-DNJ on α-amylase and maltase. Third,

based on the concentrations of iminosugar in the mulberry leaves and fruits and inhibitory effects of mulberry leaf and fruit extracts on α-amylase and α-glucosidase activities, contribution of each iminosugar to the inhibition should be clarified.

In this thesis, we examined the inhibition of α-amylase and α-glucosidase by components of *M. australis*. In Chapter 2, we quantified the concentrations of iminosugar in *M. australis* leaves. In Chapter 3, we made kinetic analysis of *M. australis* leaf extracts and iminosugar on α-amylase and α-glucosidase activities. In Chapter 4, we quantified the concentrations of iminosugar, anthocyanin, and glucose in *M. australis* fruits. In Chapter 5, we made kinetic analysis of *M. australis* fruit extracts, iminosugar, anthocyanin, and glucose, on α-amylase and α-glucosidase activities.

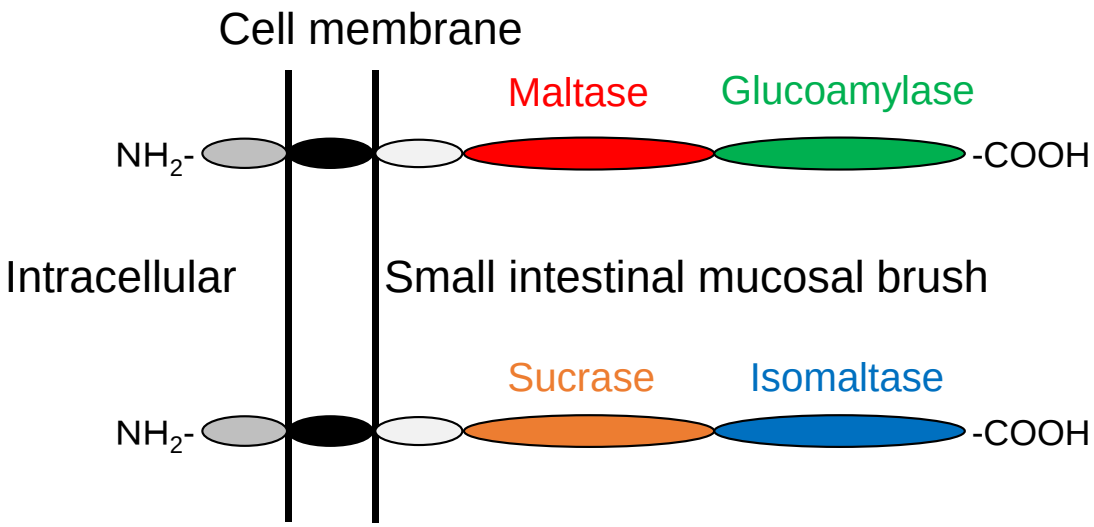

Fig. 1-1. Structure of α-glucosidase.

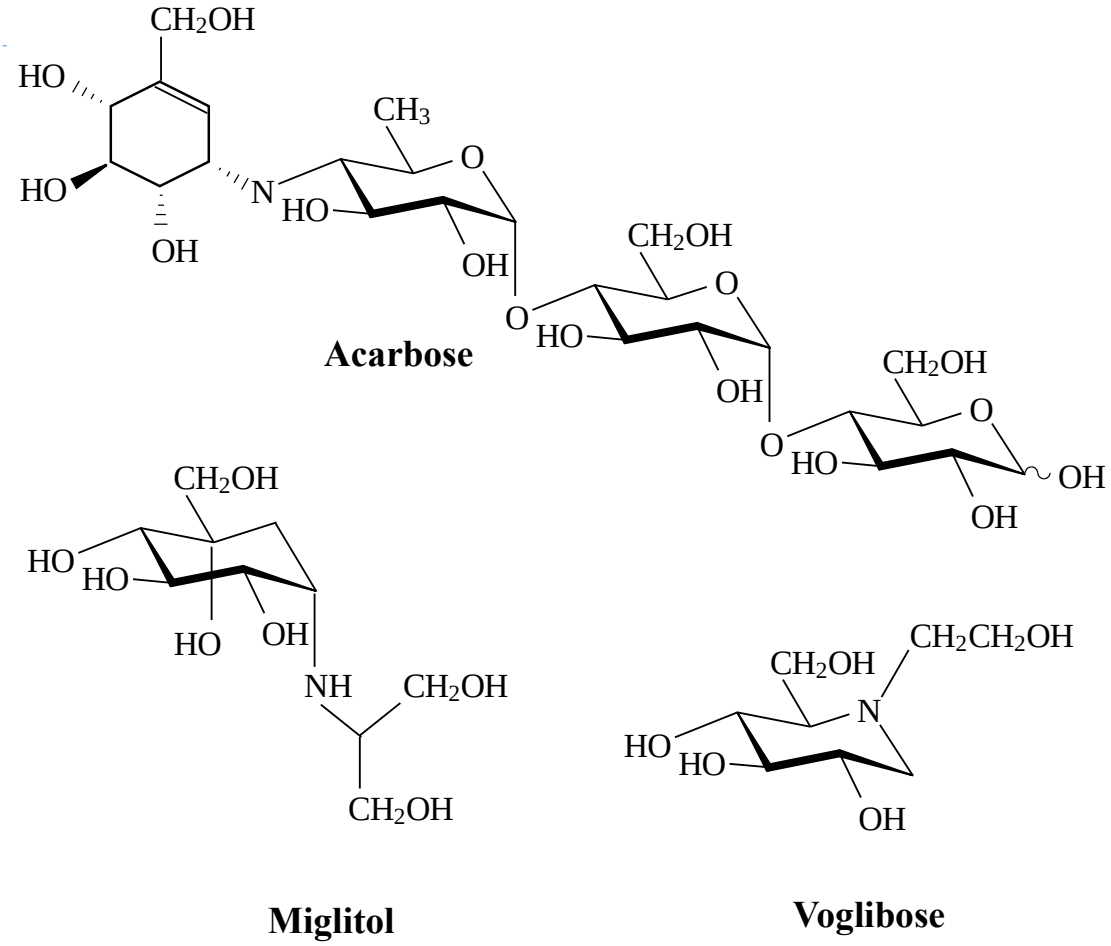

Fig. 1-2. Structures of Acarbose, Miglitol, and Voglibose.

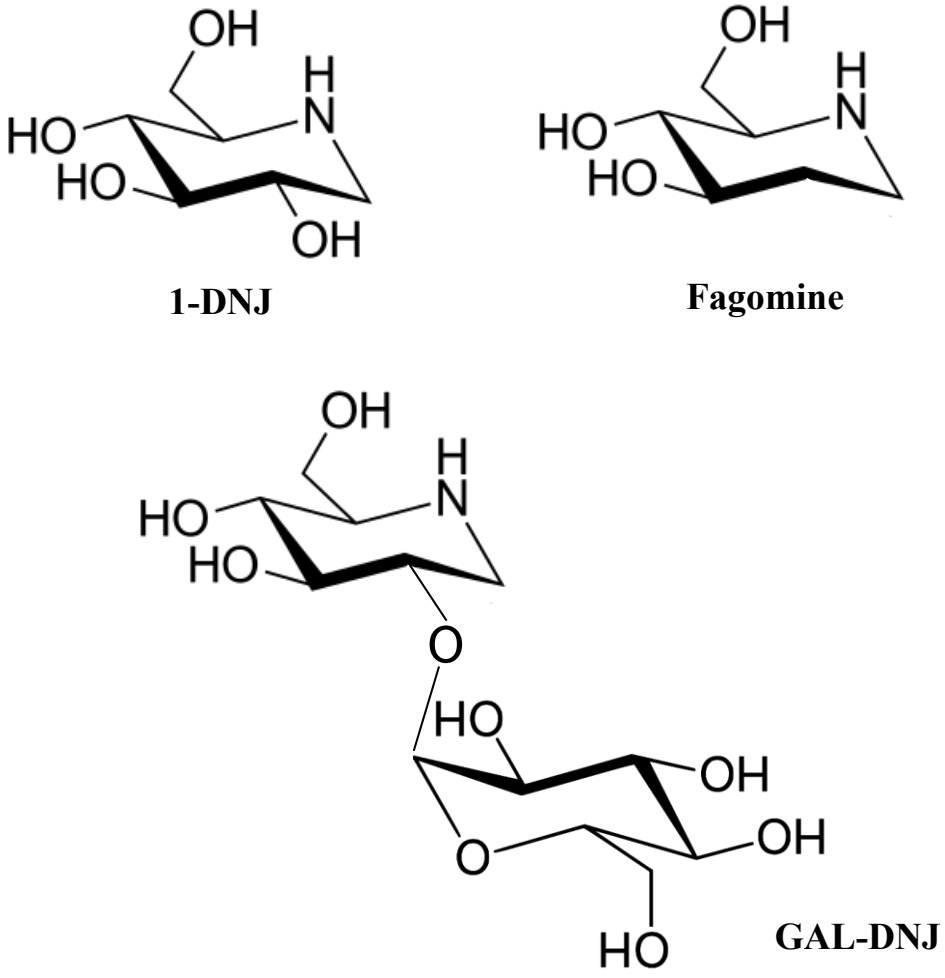

Fig. 1-3. Structures of 1-DNJ, fagomine, and GAL-DNJ.

Chapter 2

Quantification of iminosugar in *Morus australis* leaf extract*

Introduction

It was reported that the 1-DNJ concentrations in *M. australis* leaves were four to ninefold higher than those in *Morus alba* leaves distributed in Japanese mainland (39). Powdered and roasted *M. australis* leaves were developed as a tea with a function to inhibit α-glucosidase activity. The ingestion of the tea suppressed sucrose-induced elevation of blood glucose and insulin levels, indicating that the tea inhibited the postprandial elevation of blood glucose (19). In this study, we selected three iminosugars 1-DNJ, fagomine, and GAL-DNJ as a research target.

Although there are many methods for the determination of iminosugars, such as 9-fluorenylmethyl chloroformate followed by reversed-phase HPLC with UV or fluorescence detection (40), HPLC with pulsed amperometric detection (41), by far the most accurate and simple method is LC-MS/MS. In this study, we quantified their concentrations in *M. australis* leaves by LC-MS/MS.

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Qiao, Y., Nakayama, J., Iketuchi, T., Ito, M., Kimura, T., Kojima, K., Takita, T., and Yasukawa, K. (2020) Kinetic analysis of inhibition of α-glucosidase by leaf powder from *Morus australis* and its component iminosugars. *Biosci. Biotechnol. Biochem.*, **84**, 2149–2156

Materials and Methods

Materials –1-DNJ was purchased from Fujifilm Wako Pure Chemical (Osaka, Japan) Fagomine and GAL-DNJ purified from mulberry leaves (24) were given by Dr. Toshiyuki Kimura, National Agriculture and Food Research Organization (NARO), Tsukuba, Japan. Powdered and roasted *M. australis* leaves were purchased from Urasoe-shi silver human resources center (Urasoe, Japan).

Preparation of extracts of M. australis leaves – Briefly, 50 mg of powdered or roasted *M. australis* leaves were dissolved in 5.0 mL of 50% ethanol. After sonication for 5 min, the mixture was centrifuged at 10,000 x g for 5 min at 4ºC. The supernatant was separated and filtered (0.2 μm pore size; Sartorius Stedim Biotech, Goettingen, Germany). The filtrate was diluted with 0.1% formic acid, 50% acetonitrile to be 1% v/v for the quantification of 1-DNJ and GAL-DNJ and to be 10% for the quantification of fagomine. Standard 1-DNJ, fagomine, and GAL-DNJ were dissolved in 0.1% formic acid, 50% acetonitrile to be 200–1,000 ng/mL.

LC-MS/MS analysis – LC-MS/MS analysis was performed according to Kimura et al. (24). Conditions of LC-MS/MS are as follows: apparatus, LC-20A and LCMS-8045 (Shimadzu, Kyoto, Japan); column, TSK gel Amide-80 (particle size 5 μm; 100 mm × 2.0 mm i.d., Tosoh, Tokyo, Japan); column oven temperature, 40ºC; mobile phase, 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B); mobile phase flow rate, 0.2 mL/min; injection volume, 5 μL; ion source, electrospray ionization (positive mode); drying gas, nitrogen (180ºC, 7 L/min); nebulizing gas, nitrogen (1.6 bar); capillary voltage, −4,500 V; hexapole RF, 100 Vpp; quadrupole ion energy, 5 eV; collision gas, nitrogen (1.6 bar); collision energy, 10 eV; collision RF, 100 Vpp; and mass range, m/z

50–1500. Elution gradients are as follows: 0–2.0 min, 20–60% B; 2.0–5.5 min, 60% B; 5.5–5.6 min, 60–20% B; and 5.6–8.0 min, 20% B.

1-DNJ, fagomine, and GAL-DNJ were detected individually in the post column by MS/MS with multiple reaction monitoring (MRM) for transition of the parent ions to the product ions. The concentrations of 1-DNJ, fagomine, and GAL-DNJ in powdered and roasted *M. australis* leaf extracts were calculated from calibration curves using standard 1-DNJ, fagomine, and GAL-DNJ.

Results and Discussion

Quantification of iminosugars in M. australis leaves by LC-MS/MS – For quantification of iminosugars in *M. australis* leaves, we applied a standard 1-DNJ, fagomine, and GAL-DNJ to LC-MS/MS to make a calibration curve (Fig. 2-1). The total ion scanning profile showed an intense molecular ion at m/ z 164.20 $[M+H]^+$ for 1-DNJ, 148.20 $[M+H]^+$ for fagomine, and 326.20 $[M+H]^+$ for GAL-DNJ. The product ion scanning for these ions showed intense molecular ions at m/z 69.20 $[M+H-95]^+$, 80.20 $[M+H-84]^+$, and 146.20 $[M+H-H_2O]^+$ for 1-DNJ (Fig. 2-2A), at m/z 86.15 $[M+H-62]^+$, 112.15 $[M+H-2H_2O]^+$, and 130.15 $[M+H-H_2O]^+$ for fagomine (Fig. 2-2B), and at m/z 61.10 $[M+H-265]^+$, 146.15 $[M+H-180]^+$, and 164.20 $[M+H-162]^+$ for GAL-DNJ (Fig. 2-2C). The MRM chromatogram of the total of these three ions showed a peak at 5.4 min for 1-DNJ (Fig. 2-3A), at 4.0 min for fagomine (Fig. 2-3B), and at 4.3 min for GAL-DNJ (Fig. 2-3C). The peak intensity increased linearly with increasing concentration of 1-DNJ (0–1,000 ng/mL) (Fig. 2-1A), fagomine (0–1,000 ng/mL) (Fig. 2-1B) or GAL-DNJ (0–1,000 ng/mL) (Fig. 2-1C).

We applied the extracts of powdered or roasted *M. australis* leaves to determine the

concentrations of 1-DNJ, fagomine, and GAL-DNJ in the extracts of powdered (Fig. 2-4A–C) and roasted (Fig. 2-4D–F) leaves. The MRM chromatogram showed one peak at 5.4 min for the analysis of 1-DNJ (Fig. 2-4A, D), two peaks at 3.4 and 4.0 min, respectively, for the analysis of fagomine (Fig. 2-4B, E), and one peak at 4.3 min for the analysis of GAL-DNJ (Fig. 2-4C, F). In the analysis of fagomine, the peak at 4.0 min was used (Fig. 2-4B, E), according to the MRM chromatogram of standard fagomine (Fig. 2-3B). The structure of the substance corresponding to the peak at 3.4 min is unknown. Based on the calibration curves (Fig. 2-1), the concentrations of 1-DNJ, fagomine, and GAL-DNJ in the powdered leaf extracts were calculated to be 40, 4.6, and 25 μg/mL, respectively, and those in the roasted leaf extracts were calculated to be 10, 2.4, and 7.3 μg/mL, respectively. Thus, the concentrations of these three iminosugars in the leaves were calculated to be 4.0, 0.46, and 2.5 mg/g, respectively, for the powdered ones and 1.0, 0.24, and 0.73 mg/g, respectively, for the roasted ones. These results indicated that the iminosugar concentrations in the roasted leaves were 25–52% of those in the powdered leaves, suggesting that the roasting process considerably degraded iminosugars. These results also indicated that the 1-DNJ concentration in the leaves was 1.6–8.7 and 1.4–4.2 fold higher than the fagomine and GAL-DNJ concentrations, respectively. The 1-DNJ concentrations of mulberry leaves in *M. alba* and *M. bombysis* were reported to be 1.0–1.4 mg/g and those of mulberry leaf products on the market were 1.3–4.8 mg/g (24). Thus, it seems that the 1-DNJ concentration of *M. australis* leaf is relatively high. However, it should be noted that 1-DNJ concentration in mulberry leaf varies depending on seasons and collection site.

Conclusion – By combining LC-MS/MS, the kinetic analysis of leaf extracts and its components might be valuable to evaluate the inhibitory activity toward α-glucosidase of

various plant products.

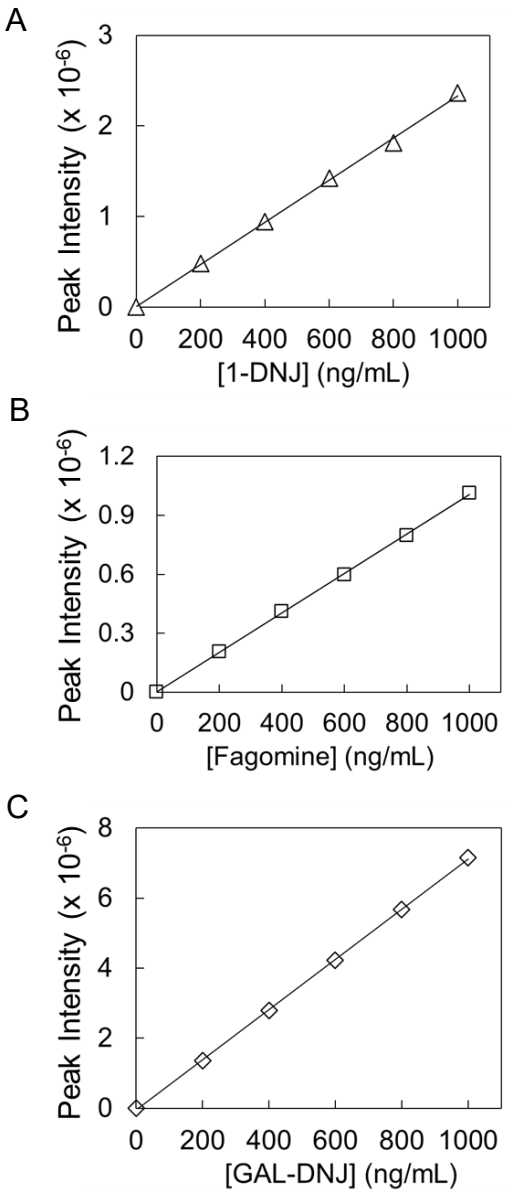

Fig. 2-1. Calibration curves of 1-DNJ, fagomine, and GAL-DNJ. Calibration curves of 1-DNJ (0–1000 ng/mL) (A), fagomine (0–1000 ng/mL) (B), and GAL-DNJ (0–1000 ng/mL) (C) are shown.

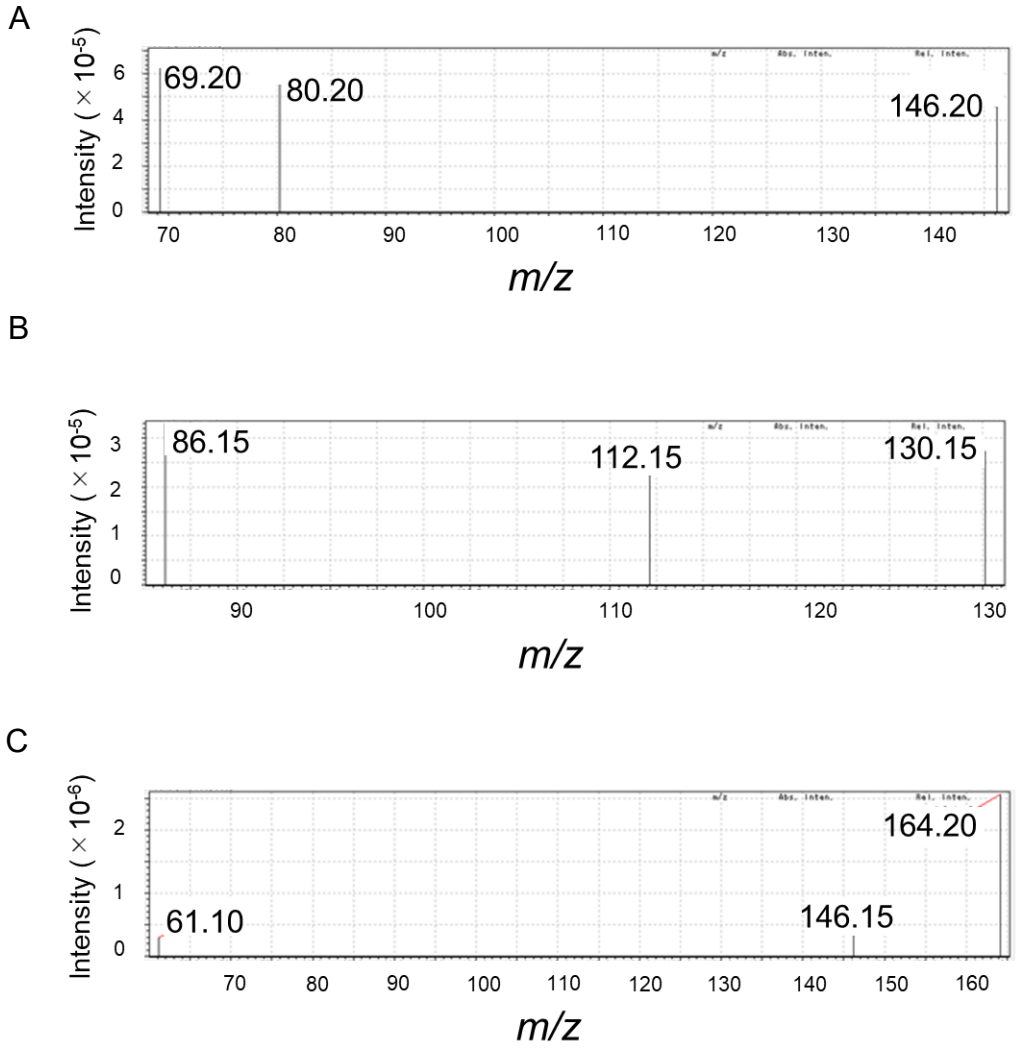

Fig. 2-2. Product ion spectra of 1-DNJ, fagomine, and GAL-DNJ. Standard 1-DNJ (5 μL in 1.0 μg/mL) (A), fagomine (5 μL in 1.0 μg/mL) (B), GAL-DNJ (5 μL in 1.0 μg/mL) (C) were applied. MRM chromatogram of the total of ions at *m/z* 69.20 $[M+H-95]^+$, 80.20 $[M+H-84]^+$, and 146.20 $[M+H-H_2O]^+$ for 1-DNJ (A), at 86.15 $[M+H-62]^+$, 112.15 $[M+H-2H_2O]^+$, and 130.15 $[M+H-H_2O]^+$ for fagomine (B), and at 61.10 $[M+H-265]^+$, 146.15 $[M+H-180]^+$, and 164.20 $[M+H-162]^+$ for GAL-DNJ (C) are shown.

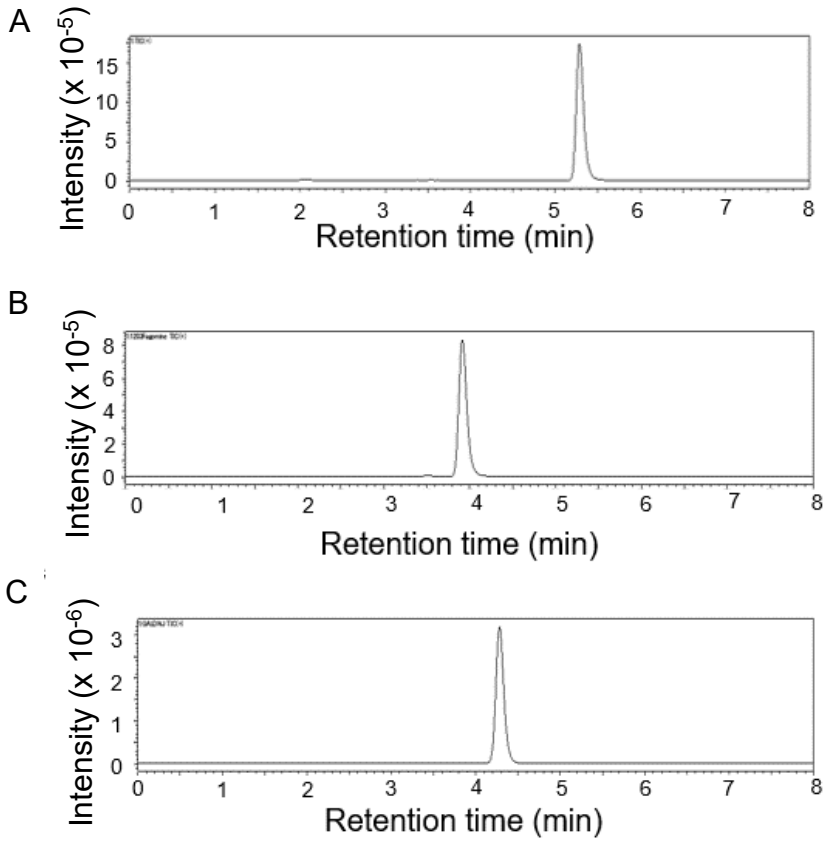

Fig. 2-3. MRM chromatogram of 1-DNJ, fagomine, and GAL-DNJ. Standard 1-DNJ (5 μL in 1.0 μg/mL) (A), fagomine (5 μL in 1.0 μg/mL) (B), and GAL-DNJ (5 μL in 1.0 μg/mL) (C) were applied.

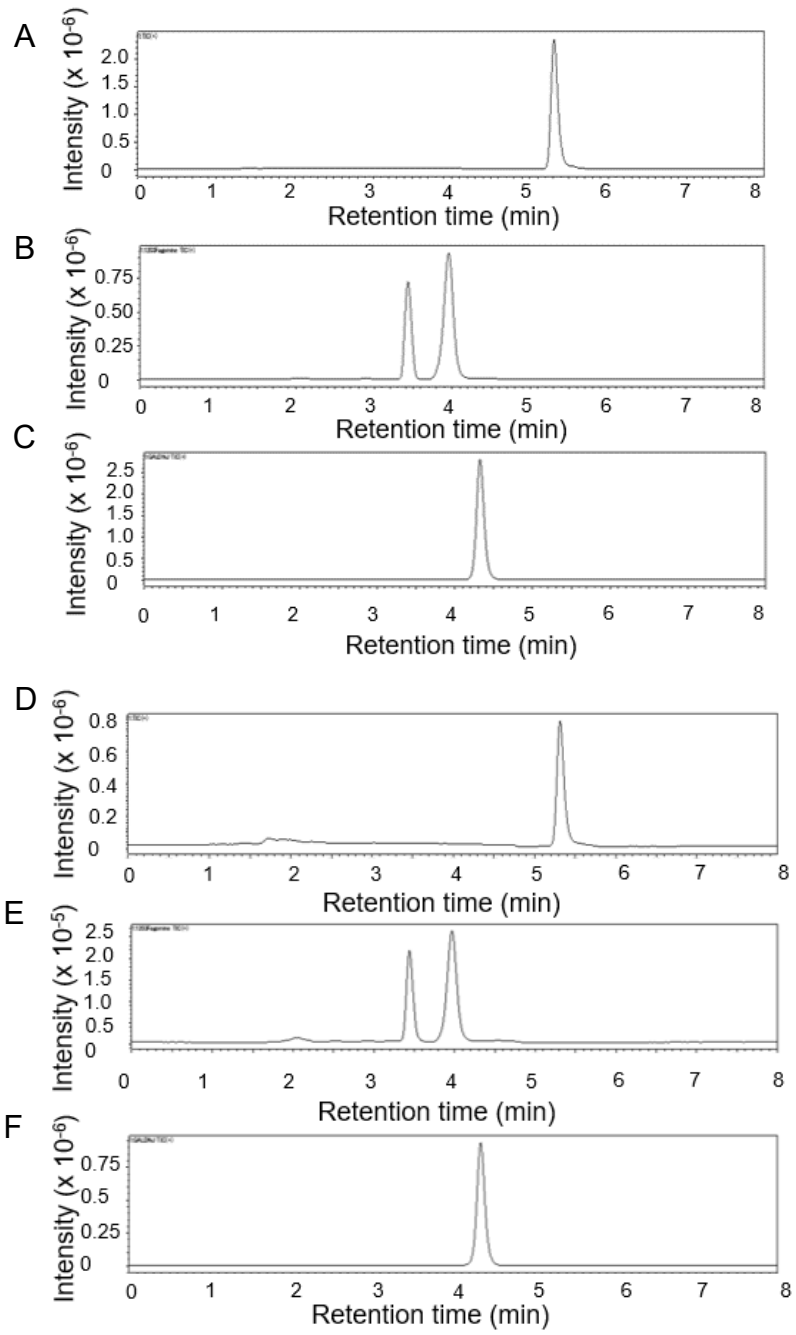

Fig. 2-4. MRM chromatogram of the extracts of *M. australis* leaves. The extracts of powdered (A–C) or roasted (D–F) leaves were applied. MRM chromatogram of the total of ions at m/z 69.20 $[M+H-95]^+$, 80.20 $[M+H-84]^+$, and 146.20 $[M+H-H_2O]^+$ for 1-DNJ (A, D), at 86.15 $[M+H-62]^+$, 112.15 $[M+H-2H_2O]^+$, and 130.15 $[M+H-H_2O]^+$ for fagomine (B, E), and at 61.10 $[M+H-265]^+$, 146.15 $[M+H-180]^+$, and 164.20 $[M+H-162]^+$ for GAL-DNJ (C, F) are shown.

Chapter 3

Kinetic analysis of inhibition of α-amylase and α-glucosidase by *Morus australis* leaf extract and its component iminosugar*

Introduction

Morus, a genus of flowering plants in the family Moraceae, is commonly known as mulberry. It is cultivated as a crop for silkworm feed, fruit, and timber. Recently, health effects of *Morus* are attracting attention. Intake of the mulberry tea inhibited postprandial elevation of blood glucose in human (18), suggesting that the tea might suppress prolonged postprandial hyperglycemia, a risk factor for type 2 diabetes. Iminosugars, such as 1-deoxynojirimycin (1-DNJ), fagomine, and 2-*O*-α-D-galactopyranosyl deoxynojirimycin (GAL-DNJ), are contained in *Morus*. These iminosugars inhibit postprandial elevation of blood glucose by inhibiting α-glucosidase (18). Above all, the contribution of 1-DNJ is the most. *Morus australis* is distributed in Ryukyu islands. In comparison with *Morus sp.* distributed in Japanese main island, *M. australis* contains more 1-DNJ (24). Kume et al. (27, 42) showed that the intake of *M. australis* tea

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*The content described in this Chapter was originally published in the following two papers

Qiao, Y., Nakayama, J., Iketuchi, T., Ito, M., Kimura, T., Kojima, K., Takita, T., and Yasukawa, K. (2020) Kinetic analysis of inhibition of α-glucosidase by leaf powder from *Morus australis* and its component iminosugars. *Biosci. Biotechnol. Biochem.*, **84**, 2149–2156

Qiao, Y., Ito, M., Kimura, T., Ikeuchi, T., Takita, T., and Yasukawa, K. (2021) Inhibitory effect of *Morus australis* leaf extract and its component iminosugars on intestinal carbohydrate-digesting enzymes. *J. Biosci. Bioeng.*, **132**, 226–233

suppressed sucrose-induced elevation of blood glucose and insulin levels in human.

α-Glucosidase includes not only maltase but also glucoamylase, sucrase, and isomaltase (2). In addition, α-amylase [EC 3.2.1.1] as well as α-glucosidase is deeply involved in carbohydrate digestion. To pursue the application of the intake of mulberry tea, the inhibitory effects of the extract of mulberry leaf and its component iminosugars against these carbohydrate digestion enzymes should be analyzed.

Health foods, such as indigestible dextrin is a type of dietary fiber that not only modulates intestinal functions but also decreases serum cholesterol and triacylglycerol (43), increased the length of small intestinal villi (44). Simultaneous intake of these food components could be expected to produce additive or synergistic benefits in decreasing risk factors for lifestyle-related diseases (45).

In chapter 2, we quantified the concentrations of 1-DNJ, fagomine, and GAL-DNJ in the boiled extracts of powdered and roasted *M. australis* leaves by LC-MS/MS. In this study, we made a kinetic analysis of the inhibitory effects of the *M. australis* leaf extracts and iminosugars against α-amylase, maltase, glucoamylase, sucrase, and isomaltase. We also examined the effects of indigestible dextrin on these enzymes.

Materials and Methods

Preparation of enzyme solution – α-Amylase from human saliva (Sigma-Aldrich, St. Louis, MO, USA) (5 mg) was dissolved in 0.5 mL of water to be 10 mg/mL (69 μg protein/mL). Rat intestinal acetone powder (Sasaki Chemical, Kyoto, Japan) (0.5 g) was suspended with 5 mL of water followed by sonication. The solution was centrifuged at 10,000 g for 15 min at 4ºC, and the supernatant was collected. Then, the solution was diluted with water to be 70 mg/mL (2.1 mg protein/mL) and used as the α-glucosidase

solution that contains maltase, sucrase, glucoamylase, and isomaltase. Protein concentration was determined using Protein Assay CBB Solution (Nacalai Tesque, Kyoto, Japan) with bovine serum albumin (Nacalai Tesque) as a standard.

Preparation of iminosugar and indigestible dextrin solutions – 1-DNJ was purchased from Fujifilm Wako Pure Chemical. Fagomine and GAL-DNJ were purified. Fagomine and GAL-DNJ purified from mulberry leaves (24) were given by Dr. Toshiyuki Kimura, National Agriculture and Food Research Organization (NARO), Tsukuba, Japan. Powdered and roasted *M. australis* leaves were purchased from Urasoe-shi silver human resources center (Urasoe, Japan). Indigestible dextrin was purchased from Matsutani Chemical Industry (Itami, Japan). 1-DNJ, fagomine, GAL-DNJ, and indigestible dextrin were dissolved in water.

Preparation of M. australis leaf extracts – Powdered and roasted *M. australis* leaves were purchased from Urasoe-shi Silver Human Resources Center (Urasoe, Japan). Boiled water (50 mL) was added to powdered or roasted *M. australis* leaves (500 mg) and stirred for 5 min. The solution was suction-filtrated (0.7 mm cellulose filter paper, Whatman, Middlesex, UK), and the filtrate was collected and used as the leaf extract.

Preparation of powdered M. australis leaf extracts blended with indigestible dextrin – Powdered *M. australis* leaf extract and indigestible dextrin were mixed in the ration of 3 to 1 in weight. Boiled water (50 mL) was added to the mixture (2 g) and stirred for 5 min. The solution was suction-filtrated, and the filtrate was collected and used as the leaf extract blended with indigestible dextrin.

Measurement of α-amylase activity – Pre-incubation (90 μL) was initiated by mixing 10 μL of 20% w/v soluble starch (Nacalai Tesque) in 70 μL 100 mM phosphate buffer

(pH 6.0) and 10 μL of the extract of powdered or roasted *M. australis* leaves or 10 μL of the 1-DNJ, fagomine, GAL-DNJ, or indigestible dextrin solution in water. After the pre-incubation at 37ºC for 10 min, the reaction was initiated by adding 10 μL of 0.2 mg/mL (14 μg protein/mL) α-amylase solution. After 2 min, 100 μL of DNS solution (0.5% w/v 3,5-dinitrosalicylic acid (Nacalai Tesque), 1.6% w/v NaOH, 30% w/v potassium sodium tartrate) was added to the reaction solution. The solution was incubated at 100ºC for 15 min and at 0ºC for 5 min. Then, the absorbance at 540 nm (A540) was measured with an EnSight multimodal plate reader (PerkinElmer, Waltham, MA, USA). The initial reaction rate was estimated from the time-course of the production of reducing sugars with maltose as a standard.

Measurement of maltase, sucrase and isomaltase activities – Sucrose and isomaltose were purchased from Fujifilm Wako Pure Chemical and Tokyo Kasei (Tokyo, Japan), respectively. For measurement of maltase, sucrase and isomaltase activities, pre-incubation (90 μL) was initiated by mixing 70 μL of 0.1 M phosphate buffer (pH 6.0), 10 μL of sucrose or isomaltose solution, and 10 μL of the extract of powdered or roasted *M. australis* leaves or 10 μL of the 1-DNJ, fagomine, GAL-DNJ, or indigestible dextrin solution in water. After the pre-incubation at 37ºC for 10 min, the reaction was initiated by adding 10 μL of 35 mg/mL (1.0 mg protein/mL) α-glucosidase solution for maltase or 70 mg/mL (2.1 mg protein/mL) α-glucosidase solution for sucrase and isomaltase, and continued at 37ºC. Blank solution was prepared by adding 10 μL of 0.1 M phosphate buffer (pH 6.0). After 2 min, the solution was boiled for 5 min to stop reaction. To 13.3 μL of the maltase solution, 200 μL of the coloring solution in Glucose CII test (Fujifilm Wako Pure Chemical) was added. To 100 μL of the sucrase and isomaltase solution, 100 μL of the coloring solution in Glucose CII test was added. The A505 was measured with

an EnSight.

Measurement of glucoamylase activity – Glucoamylase and α-glucosidase assay kit was purchased from Kikkoman Corporation (Noda, Japan). For measurement of glucoamylase (starch hydrolase releasing β-glucose from the non-reducing ends of starch) activity (A) and other α-glucosidase (aryl-glucosides hydrolase releasing β-glucose) activity (B), pre-incubation (110 μL) was initiated by mixing 50 μL of β-glucosidase in water, 50 μL of G2-β-PNP in water, and 10 μL of the extract, iminosugar, or indigestible dextrin solution in water. For measurement of B, pre-incubation (210 μL) was initiated by mixing 200 μL of PNPG in water, and 10 μL of the extract, iminosugar, or indigestible dextrin solution in water. After the pre-incubation at 37ºC for 10 min, the reaction was initiated by adding 10 μL of 35 mg/mL (1.0 mg protein/mL) α-glucosidase solution and continued at 37ºC. After 10 min, the $NaCO_3$ solution (100 or 200 μL) was added for 5 min to stop reaction. The A400 was measured with an EnSight, and the reaction rate for A + B and that for B were obtained, respectively. The reaction rate for A was calculated by reducing B from A + B.

Steady-state kinetic analysis – From the reaction rate, the Michaelis constant in the absence of inhibitor (K_m), the Michaelis constant in the presence of inhibitor (K_{mapp}), and maximum velocity (V_{max}) were calculated based on using Hanes-Woolf plot with Microsoft Excel. The inhibition constant (K_i) of reversible competitive inhibitor was calculated by the following equation:

$$\frac{K_{mapp}}{K_m} = 1 + \frac{[I]_o}{K_i} \quad (1)$$

where $[I]_o$ is the initial inhibitor concentration.

Thermodynamic analysis – The enthalpy change, ΔH^o, of the dissociation of the complex of α-glucosidase and inhibitor was determined from a van't Hoff plot according to Equation 2, as described by Horn et al. (46). The Gibbs free energy change of dissociation, ΔG^o, and the entropy change of dissociation, ΔS^o, at certain temperature were determined according to Equations 3 and 4, respectively.

$$\ln(K_i) = A - (\Delta H^o/R)(1/T) \quad (2)$$

$$\Delta G^o = -RT \ln K_i \quad (3)$$

$$\Delta S^o = (\Delta H^o - \Delta G^o)/T \quad (4)$$

where A, R, and T are the constant term, the gas constant (= 8.314 J K^{-1} mol^{-1}), and absolute temperature in degrees Kelvin.

Statistical analysis – Experiments were conducted two times on different days. Values represent the average of triplicate determination. Error bars in figures indicate SD values.

Results and Discussion

Inhibition of α-amylase by the extracts of M. australis leaves and iminosugars – Figure 3-1A and B shows the reaction rates with 20 mg/mL soluble starch in the presence of varying concentrations of powdered and roasted leaf extracts, respectively, at pH 6.0 at 37 ºC. The initial reaction rates were stable with increasing concentrations of extract. Figure 3-1C and D shows the reaction rates in the presence of varying concentrations of iminosugars. The reaction rate decreased with increasing concentrations of 1-DNJ or fagomine, but did not decrease with increasing concentrations of GAL-DNJ, indicating that 1-DNJ and fagomine inhibited α-amylase while the leaf extracts and GAL-DNJ did

not inhibit it. The IC_{50} values, which were determined using intersection at 50% activity in line graphs, of 1-DNJ and fagomine were 78 mM (13 mg/mL) and 38 mM (5.6 mg/mL), respectively, which were much higher than the IC_{50} values of 1-DNJ and fagomine for maltase (4.3 μM ($7.0x10^4$ mg/mL) and 1.2 mM (0.18 mg/mL), respectively) (Table 1), indicating that their inhibitory effects against α-amylase were weaker. 1-DNJ and fagomine inhibited α-amylase while the *M. australis* leaf extract did not inhibit it. This can be explained by that the amounts of 1-DNJ and fagomine in the leaf extracts were not enough to inhibit α-amylase. The concentrations of 1-DNJ and fagomine in the powdered leaf extract were 4.0 and 0.46 mg/g, respectively, and those in the roasted leaf extract were 1.0 and 0.24 mg/g (Chapter 2). This indicated that 10 mg/mL powdered leaf contained 40 mg/mL 1-DNJ and 4.6 mg/mL fagomine, which were 1000-fold lower than the IC_{50} values (13 and 5.6 mg/mL, respectively). Acarbose strongly inhibits α-amylase (IC_{50} value 83.33 ± 0.34 μg/mL) (7), but iminosugars have a weaker inhibitory effect. Liu et al. (47) reported that neither 1-DNJ nor fagomine inhibited α-amylase by showing that the IC_{50} values were higher than 0.1 mM, which agreed with our results.

Kinetic analysis revealed that the inhibition was competitive. The K_m values were 5.5 mg/mL in the absence of iminosugar and 34 and 33 mg/mL in the presence of 1-DNJ and fagomine, respectively, and the K_i values of 1-DNJ and fagomine calculated using Eq.1 were 15 and 8.0 mM (2.5 and 1.1 mg/mL), respectively (Table 2). Compared with the K_i values of glucose and maltose for wheat β-amylase at pH 5.4 at 25ºC (390 and 160 mM, respectively) (30), the K_i values of 1-DNJ and fagomine were 10–50 fold lower, suggesting that 1-DNJ and fagomine bind any of the subsites of α-amylase and inhibit the activity.

Inhibition of α-glucosidase *by the extracts of M. australis leaves and iminosugars* – We measured the inhibition of the extracts of *M. australis* leaves and iminosugars against the maltase-mediated activity of rat intestinal acetone powder, in which the IC_{50} values of the extracts (50 mL) of powdered and roasted leaves (500 mg) were 0.36 and 1.1 mg/mL, respectively, and those of 1-DNJ, fagomine, and GAL-DNJ were 4.3 x 10^3, 1.2, and 8.9 mM (7.0 x 10^4, 0.18, and 2.9 mg/mL), respectively (Table 1), indicating that the inhibitory effects of 1-DNJ were 280 and 2100 fold higher than those of fagomine and GAL-DNJ, respectively.

We examined the inhibitory effects against glucoamylase-, sucrase-, and isomaltase-mediated activities of rat intestinal acetone powder. Figure 3-2A, D, and G show the reaction rates in the hydrolysis of 15 mM G2-β-PNP, 100 mM sucrose, or 50 mM isomaltose, respectively, in the presence of various concentrations of the extract of powdered or roasted *M. australis* leaves at pH 6.0 at 37ºC. The reaction rate decreased with increasing the concentration of each extract. The IC_{50} values of powdered and roasted leaf extracts were 4.5 and 4.2 mg/mL for glucoamylase, 0.050 and 0.15 mg/mL, respectively, for sucrase, 0.24 and 0.58 mg/mL for isomaltase. This indicated that the inhibitory effects against sucrase and isomaltase of the powdered leaf extract were higher than those of the roasted leaf extract, respectively, while those against glucoamylase of the powdered and roasted leaf extracts were almost the same. Figure 3-2B–I shows the reaction rates of α-glucosidase in the rat intestinal acetone powder in the hydrolysis of 15 mM G2-β-PNP (Fig. 3-2B, C), 100 mM sucrose (Fig. 3-2E, F), or 50 mM isomaltose (Fig. 3-2H, I) in the presence of various concentrations of 1-DNJ, fagomine, and GAL-DNJ at pH 6.0 at 37ºC. The reaction rates decreased with increasing concentrations of each iminosugar.

The IC_{50} values of 1-DNJ, fagomine, and GAL-DNJ for maltose, glucoamylase, sucrase, and isomaltase are shown in Table 1, indicating that the order of inhibitory effects was 1-DNJ > fagomine > GAL-DNJ, and the inhibitory effects of 1-DNJ were 19–620 and 800–2100 fold higher than those of fagomine and GAL-DNJ, respectively. Our results indicated that the α-glucosidase inhibitory effect of 1-DNJ was significantly higher than that of fagomine and GAL-DNJ. According to Asano (15), reversible-glucosidase inhibitors often have structures closely resembling glucose. α-Glucosidase has a negatively charged region in its catalytic center due to the presence of highly conserved acidic amino acid residues that are necessary for enzyme activity (48), suggesting that iminosugars are protonated at its nitrogen atom resulting in a positive charge that interacts tightly with the negatively charged residues in the active site. Both 1-DNJ and fagomine satisfy the above conjecture. According to Rossi et al. (49), positive charge may be important in stabilizing active site interactions, the ring size and OH group also affect binding in the enzyme active site. Therefore, the significantly strong inhibitory activity of 1-DNJ might be related to its glucose analog structure, nitrogen atom with a positive charge, ring size and OH group.

However, according to a recent study about the intestinal absorption and tissue distribution of iminosugars in rats, 1-DNJ was absorbed from the small intestine into the bloodstream faster than fagomine and GAL-DNJ, suggesting that fagomine and GAL-DNJ could be involved in α-glucosidase inhibition in the small intestine for longer than 1-DNJ (50). Interestingly, the oral intake of GAL-DNJ led to the appearance of 1-DNJ, as well as GAL-DNJ, in the plasma, suggesting that GAL-DNJ is degraded into galactose and 1-DNJ (50). Another report showed that orally-administered fagomine in rats was partly absorbed but mostly excreted in feces within 24 h (25). Therefore, in vivo studies

would also be necessary to estimate the α-glucosidase inhibitory effect of each iminosugar in the intestine.

Kinetic analysis of inhibition of α-glucosidase *by the extracts of M. australis leaves and iminosugars* – Figure 3-3A, C, and E shows the reaction rates in the hydrolysis of various concentrations of G2-β-PNP, sucrose, or isomaltose in the rat intestinal acetone powder, respectively, in the absence and presence of the extract of powdered or roasted *M. australis* leaves at pH 6.0 at 37ºC. All plots showed saturated profiles. Figure 3-4A, C, and E show Hanes Woolf plot. In Fig. 3-4A, plots showed unparallel lines, indicating mixed inhibition for glucoamylase. In Fig. 3-4C and E, plot shows parallel lines, indicating competitive inhibition for sucrase and isomaltase. The difference in the inhibitory manner might be explained by that polyphenol as well as iminosugar contained in the powdered and roasted *M. australis* leaves inhibited glucoamylase. In the hydrolysis of G2-β-PNP, the K_m values were calculated to be 4.0 mM in the absence of the extracts, 8.6 and 6.3 mM in the presence of powdered and roasted extracts, respectively. In the hydrolysis of sucrose, they were 19 mM in the absence of the extracts and 65 and 90 mM in the presence of powdered and roasted extracts, respectively. In the hydrolysis of isomaltose, they were 8.9 mM in the absence of the extracts and 50 and 42 mM in the presence of powdered and roasted extracts, respectively. The K_i values of powdered and roasted extracts calculated using Eq. 1 were 0.021 and 0.040 mg/ mL, respectively, for sucrase, and 0.052 and 0.16 mg/mL, respectively, for isomaltase.

Figure 3-3B, D, and F show the reaction rates in the hydrolysis of various concentrations of G2-β-PNP, sucrose, or isomaltose in the rat intestinal acetone powder, respectively, in the absence and presence of iminosugar at pH 6.0 at 37ºC. All plots showed saturated profiles. Figure 3-4B, D, and F show Hanes Woolf plot, indicating

competitive inhibition. The K_m value of 1-DNJ was 18 mM (8.3 mg/mL), and those of fagomine and GAL-DNJ were not obtained in the hydrolysis of G2-β-PNP. The K_m values of 1-DNJ, fagomine, and GAL-DNJ were 108, 129, and 151 mM (37, 44, and 52 mg/mL), respectively, in the hydrolysis of sucrose, and 43, 42, and 30 mM (15, 14, and 10 mg/mL), respectively, in the presence of isomaltose. The K_i values of 1-DNJ (molecular weight: 163), fagomine (147), and GAL-DNJ (325) for glucoamylase, sucrase, and isomaltase are shown in Table 2, indicating that the order of K_i values was 1-DNJ < fagomine < GAL-DNJ.

Inhibition of α-amylase and α-glucosidase by indigestible dextrin – Dextrin is a mixture of low-molecular-weight D-glucose polymers with a-1,4 and a-1,6 bonds. Dextrin is produced by the hydrolysis of starch or glycogen. Indigestible dextrin is a water-soluble dextrin, which is produced by the extraction of dietary fiber. Dextrin and indigestible dextrin are used as a food ingredient (51). We analyzed the inhibitory effects of indigestible dextrin against α-amylase and rat intestinal acetone powder-mediated maltase, glucoamylase, sucrase, and isomaltase.

Figure 3-5A shows the reaction rates of α-amylase in the hydrolysis of 20 mg/mL soluble starch in the presence of various concentrations of indigestible dextrin at pH 6.0 at 37ºC. The reaction rates decreased with increasing the concentration of indigestible dextrin with the IC_{50} values of 14 mg/mL (Table 1). Figure 3-5B and C show the reaction rates in the hydrolysis of 50 mM maltose or 15 mM G2-β-PNP, respectively. The reaction rates slightly decreased or were almost stable with increasing concentrations of indigestible dextrin. Figure 3-5D and E show the reaction rates in the hydrolysis of 100 mM sucrose or 50 mM isomaltose, respectively. The reaction rates decreased with increasing the concentration of indigestible dextrin with the IC_{50} values of 2.5 and 2.3

mg/mL, respectively (Table 1), corresponding to approximately 1 mM on the assumption that the molecular weight is around 2000. These results indicated that indigestible dextrin inhibited α-amylase, sucrase, and isomaltase but did not maltase or glucoamylase. We speculate that this might be explained by that the substrates of maltase and glucoamylase are di or oligo-glucose consisting of a-1,4 linkage while those of α-amylase, sucrase, and isomaltase are not.

Inhibition of α-amylase and a-glucosidase by the powdered M. australis leaf extract blended with indigestible dextrin – We attempted to add the inhibitory effect against α-amylase to *M. australis* leaf extract by adding indigestible dextrin solution. Figure 3-6A shows the reaction rates of α-amylase in the hydrolysis of soluble starch with varying concentrations of the powdered *M. australis* leaf extract blended with indigestible dextrin. The reaction rates decreased with increasing the concentration of extract with the IC_{50} values of 23 mg/mL (Table 1). Figure 3-6B–E show the reaction rates in the hydrolysis of maltose, G2-β-PNP, sucrose, isomaltose in the rat intestinal acetone powder. The reaction rates decreased with increasing concentrations of the extract with the IC_{50} values of 0.65, 16, 0.42, and 1.2 mg/mL, respectively (Table 1). Kinetic analysis revealed that the inhibition manner for maltase, sucrase, and isomaltase were competitive (Fig. 3-7), and the K_i values were 0.030, 0.060, 0.21 mg/mL, respectively (Table 2). These results indicated that the extract of *M. australis* leaves blended with indigestible dextrin inhibited both α-amylase and all α-glucosidase-related enzymes. The optimization of mixing condition is the future subject.

Kinetic analysis of inhibition of maltase by the extracts of M. australis leaves – In this study, we characterized the inhibitory effects of the extracts of *M. australis* leaves and iminosugars toward the maltase-mediated maltose-hydrolyzing activity and regarded

the effects toward α-glucosidase activity.

We examined the reaction rates of maltase in the hydrolysis of 50 mM maltose in the presence of various concentrations of the extracts of powdered (0–1.0 mg/mL) or roasted (0–2.0 mg/mL) *M. australis* leaves at pH 6.0 at 37ºC (Fig. 3-8A). The reaction rate decreased with increasing the concentration of each extract. The IC_{50} values, which were determined using intersection at 50% activity in line graphs, of the extract of powdered leaves and that of roasted leaves were 0.36 and 1.1 mg/mL, respectively, indicating that the inhibitory effect of the powdered leaves was 3-fold higher than that of the roasted leaf.

We measured the reaction rates of maltase in the hydrolysis of various concentrations (0–75 mM) of maltose in the presence of 0.6 mg/mL of powdered or roasted leaf extract at pH 6.0 at 37ºC. All plots showed saturated profiles (Fig. 3-8B). Hanes-Woolf plot showed parallel lines intersecting at the Y-axis (Fig. 3-8C), indicating that the manner of inhibition was competitive.

Kinetic analysis of inhibition of maltase by 1-DNJ, fagomine, and GAL-DNJ – Figure 3-9A and B show the reaction rates of maltase in the hydrolysis of 50 mM maltose in the presence of various concentrations of 1-DNJ (0–10 μg/mL), fagomine (0–4 mg/mL), and GAL-DNJ (0–8 mg/mL) at pH 6.0 at 37ºC. The reaction rates decreased with increasing the concentration of each iminosugar. The activity was almost completely inhibited by 1-DNJ and fagomine, while 20% activity remained by GAL-DNJ, suggesting that the inhibition by GAL-DNJ is partial. The IC_{50} values were 0.70 μg/mL for 1-DNJ, 0.18 mg/mL for fagomine, and 2.9 mg/mL for GAL-DNJ, indicating that the inhibitory effect of 1-DNJ was 250- and 4,000- fold higher than that of fagomine or GAL-DNJ, respectively.

Figure 3-9C shows the reaction rates of α-glucosidase in the hydrolysis of various

concentrations (0–75 mM) of maltose in the presence of 0.25 μg/mL of 1-DNJ, 0.1 mg/mL of fagomine, or 1.5 mg/mL of GAL-DNJ at pH 6.0 at 37ºC. Like the case with the reaction in the presence of the leaf extracts, all plots showed saturated profiles (Fig. 3-9C). Hanes-Woolf plot showed parallel lines intersecting at the Y-axis (Fig. 3-9D), indicating competitive inhibition. The K_m value was calculated to be 1.9 mM in the absence of iminosugar, and the K_{mapp} values were calculated to be the 9.0 mM in the presence of 0.25 μg/mL 1-DNJ, 16 mM in the presence of 0.1 mg/mL fagomine, and 30 mM in the presence of 1.5 mg/mL GAL-DNJ.

The K_i values of 1-DNJ (molecular weight: 163), fagomine (147), and GAL-DNJ (330) calculated from the K_m and K_{mapp} values using Eq. 1 were 0.068 μg/mL (= 4.1×10^{-7} M), 14 μg/mL (= 9.2×10^{-5} M), and 102 μg/mL (= 3.1×10^{-4} M), respectively. The K_i values of 1-DNJ and fagomine thus obtained were similar to those previously reported (1.12×10^{-6} M for 1-DNJ and 3.24×10^{-4} M for fagomine) (47).

Insight into the contribution of 1-DNJ in M. australis leaves to the inhibition of α-glucosidase – As described above, the concentrations of IC_{50} value of the powdered leaf extract, roasted leaf extract, and 1-DNJ were 360, 1,100, and 0.70 μg/mL, respectively, and the concentrations of 1-DNJ in the powdered and roasted leaves were 4.0 and 1.0 mg/g, respectively. Nakanishi et al. showed that dependences on season of 1-DNJ amount and the α-glucosidase inhibitory activity of mulberry leaves exhibited similar profiles (52). In this study, the inhibitory effects of fagomine and GAL-DNJ to maltase were thought to be marginal compared with 1-DNJ. Assuming that 1-DNJ is a sole component that inhibits maltase in mulberry leaf, it was calculated that 360 μg/mL powdered leaf extract contained 1.4 μg/mL 1-DNJ, and 1,100 μg/mL roasted leaf extract contained 1.1 μg/mL 1-DNJ. These concentrations correspond to 200% and 150%, respectively, of the IC_{50}

value of 1-DNJ (0.70 μg/mL), suggesting that in *M. australis* , the inhibitory effects of 1-DNJ were suppressed in the powdered and roasted leaf extracts. We speculate that GAL-DNJ, which exhibited partial inhibition, might suppress the inhibitory effect of 1-DNJ.

Effects of temperature and pH on the inhibition of maltase by the extracts of M. australis leaves and 1-DNJ – To explore the role of 1-DNJ on the inhibition of maltase by the extracts of *M. australis* powdered leaves, we examined the effects of temperature (27–47ºC) and pH (6.0–8.0) on the inhibition. Figure 3-10 shows the effects of reaction temperature and pH on the maltase activity. The highest activity was observed at 62ºC (Fig. 3-10A), and the relatively high activity was observed at pH 5–8 (Fig. 3-10B), suggesting that maltase did not lose activity at pH 6.0–8.0 or at 27–47ºC. The reaction rates of maltase in the hydrolysis of various concentrations (0–75 mM) of maltose in the presence of 0.15 mg/mL of the powdered leaf extracts or 0.25 μg/mL 1-DNJ at pH 6.0–8.0, at 27–47ºC were measured. All plots showed saturated profiles.

The K_i values were calculated from Eq. 1. Figure 3-11A and B show the dependence of K_i values at pH 6.0 on reaction temperature and the dependence of K_i values at 37ºC on pH, respectively. The profiles of the extracts of powdered leaves and 1-DNJ were very similar, suggesting that 1-DNJ is a major inhibitory component in the powdered leaves.

The K_i values of the dissociation depended on temperature (Fig. 3-11A). Figure 3-11C shows van't Hoff plot of K_i values. Enthalpy changes (ΔH^o) at pH 6 were calculated from the slope to be -87.6 ± 6.4 kJ mol^{-1} for the extracts of powdered leaves and -72.8 ± 12.9 kJ mol^{-1} for 1-DNJ. The negative ΔH^o values of the dissociation indicate that the dissociation was exothermic. For 1-DNJ, Gibbs free energy change of dissociation (ΔG^o) at pH 6.0 at 37ºC were calculated be 38.1 kJ mol^{-1} for 1-DNJ, and

entropy change of dissociation (ΔS^{o}) at pH 6.0 at 37ºC were calculated to be −351.7 J mol^{-1} K^{-1} from Eq. 3. Since ΔH^{o} is negative and $-T\Delta S^{o}$ is positive, the dissociation is enthalpy-driven. The K_i values of the dissociation also depended on pH (Fig. 3-11B). This might be due to changes in ionization state of the amino acid residues of α-glucosidase involved in the binding.

Conclusion – *M. australis* leaves contain 1-DNJ a lot and strongly inhibit α-glucosidase activity. The K_i values of the extract of *M. australis* powdered leaf and 1-DNJ are temperature- and pH-dependent. *M. australis* leaf extract inhibited α-glucosidase but not α-amylase while indigestible dextrin could inhibit α-amylase. The ingestion of the *M. australis* leaf extract blended with indigestible dextrin could potentially suppress postprandial blood glucose level increase. According to Kimura et al.(18), the amount of 1-DNJ required to lower human blood glucose is 0.64 to 0.96 mg. From the LCMS results, an amount of powdered leaf extracts of about 0.2 g can lower blood sugar levels in humans.

Table 1. IC_{50} values.

	Amylase		Maltase		Glucoamylase		Sucrase		Isomaltase	
	(mM)	(mg/mL)	(mM)	(mg/mL)	(mM)	(mg/mL)	(mM)	(mg/mL)	(mM)	(mg/mL)
1-DNJ	78 (1)[a]	13 (1)	4.3×10^{-3} (1)	7.0×10^{-4} (1)	0.066 (1)	0.011 (1)	8.6×10^{-4} (1)	1.4×10^{-4} (1)	4.0×10^{-3} (1)	6.5×10^{-4} (1)
Fagomine	38 (0.49)[a]	5.6 (0.43)	1.2 (280)	0.18 (250)	41 (620)	5.9 (540)	0.24 (280)	0.035 (250)	0.075 (19)	0.011 (17)
GAL-DNJ	No inhibition	No inhibition	8.9 (2,100)[a]	2.9 (4,000)	53 (800)	17 (1,500)	1.3 (1,500)	0.41 (2,900)	4.8 (1,200)	0.91 (1,400)
Powdered leaf extract	—	No inhibition	—	0.36 (1)[b]	—	4.5 (1)	—	0.050 (1)	—	0.24 (1)
Roasted leaf extract	—	No inhibition	—	1.1 (3.1)[b]	—	4.2 (0.93)	—	0.15 (3.0)	—	0.58 (2.4)
Indigestible dextrin	—	14	—	No inhibition	—	No inhibition	—	2.5 (50)[b]	—	2.3 (9.6)
Powdered leaf extract with indigestible dextrin	—	23	—	0.65 (1.8)[b]	—	16 (3.6)	—	0.42 (8.4)	—	1.2 (5.0)

[a]Values in parenthesis indicate relative values compared to 1-DNJ.

[b]Values in parenthesis indicate relative values compared to powdered leaf extract.

Table 2. K_i values.

	Amylase		Maltase		Glucoamylase		Sucrase		Isomaltase	
	(mM)	(mg/mL)	(mM)	(mg/mL)	(mM)	(mg/mL)	(mM)	(mg/mL)	(mM)	(mg/mL)
1-DNJ	15 (1)[a]	2.5 (1)	4.1×10^{-4} (1)	6.8×10^{-5} (1)	0.019	0.0032	1.9×10^{-4} (1)	3.2×10^{-5} (1)	1.1×10^{-3} (1)	1.7×10^{-4} (1)
Fagomine	8.0 (0.53)[a]	1.1 (0.44)	0.092 (220)	0.014 (210)	—	ND	0.042 (220)	6.1×10^{-3} (190)	0.020 (18)	3.0×10^{-3} (18)
GAL-DNJ	No inhibition	No inhibition	0.31 (760)[a]	0.10 (1,500)	—	ND	0.18 (950)	0.059 (1,800)	2.0 (1,800)	0.65 (3,800)
Powdered leaf extract	—	No inhibition	—	0.040 (1)[b]	—	ND	—	0.021 (1)	—	0.052 (1)
Roasted leaf extract	—	No inhibition	—	0.071 (1.8)[b]	—	ND	—	0.040 (1.9)	—	0.16 (3.1)
Indigestible dextrin	—	ND	—	No inhibition	—	ND	—	ND	—	ND
Powdered leaf extract with indigestible dextrin	—	ND	—	0.030 (0.75)[b]	—	ND	—	0.060 (2.8)	—	0.21 (4.0)

[a]Values in parenthesis indicate relative values compared to 1-DNJ.

[b]Values in parenthesis indicate relative values compared to powdered leaf extract.

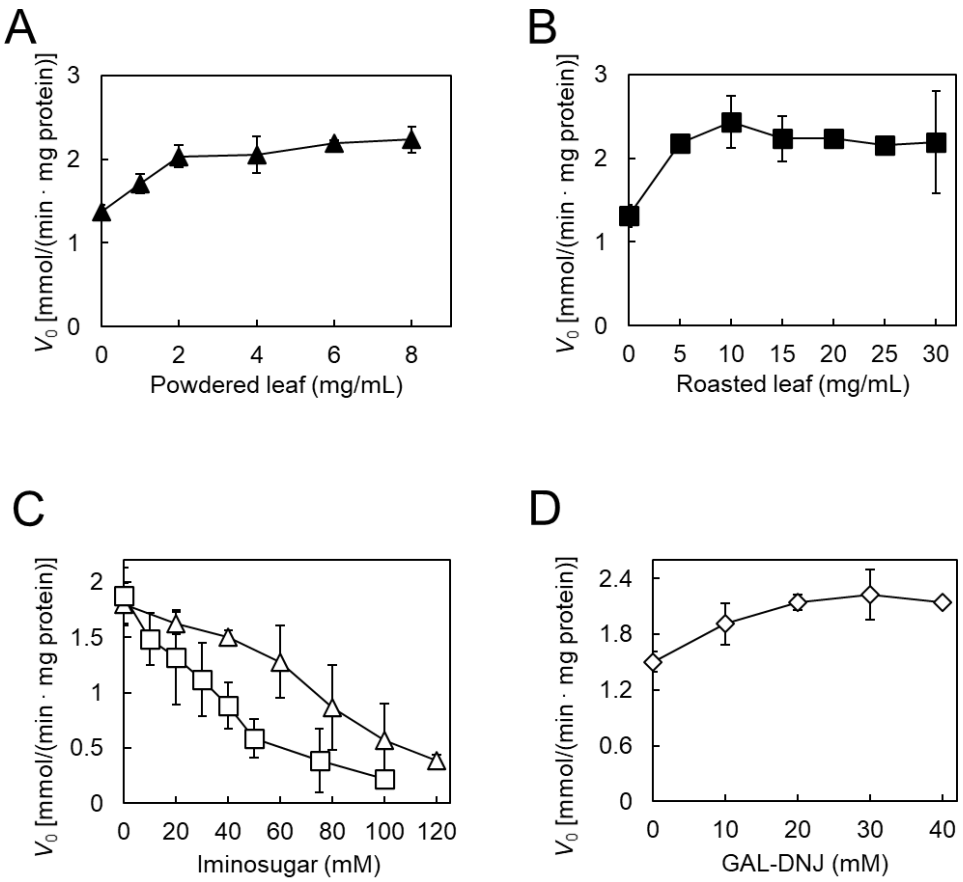
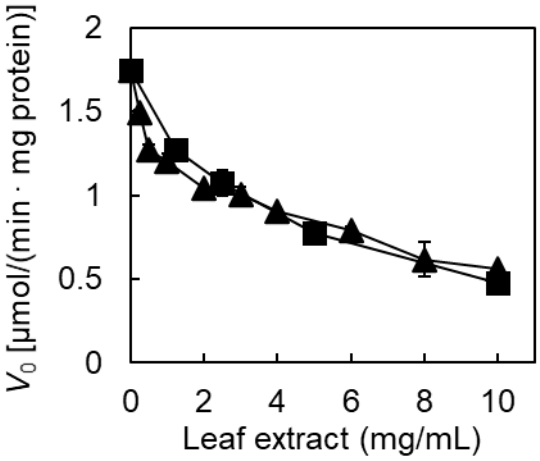
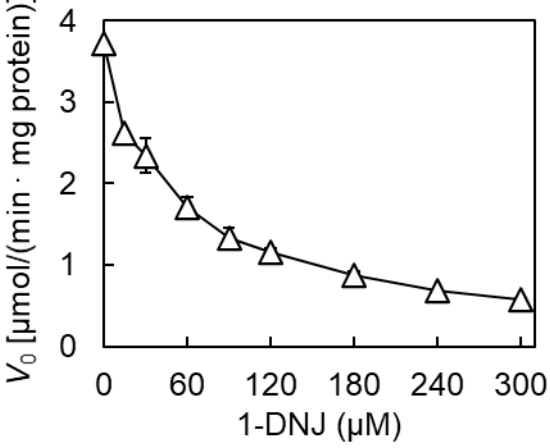


Fig. 3-1. Dependence on the extract and iminosugar concentrations of the reaction rate for the hydrolysis by human α-amylase. The reaction was carried out with 1.4 μg protein/mL human α-amylase and 20 mg/mL soluble starch in the presence of varying concentrations of powdered (A) or roasted leaf extract (B), 1-DNJ (open triangle) (C), fagomine (open square) (C), or GAL-DNJ (D) at pH 6.0 at 37 ºC. Error bars indicate SD values. The average of triplicate determination is shown.

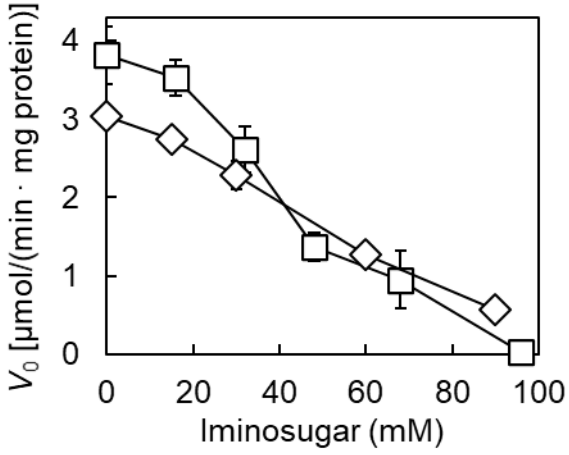
A


2
1.5
1
0.5
0
V_0 [μmol/(min · mg protein)]
0
2
4
6
8
10
Leaf extract (mg/mL)

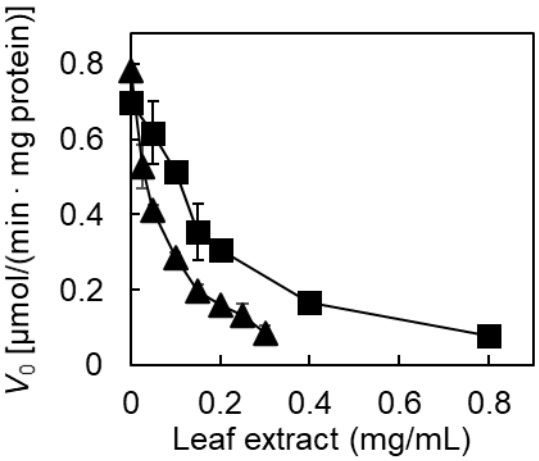
B


4
3
2
1
0
V_0 [μmol/(min · mg protein)]
0
60
120
180
240
300
1-DNJ (μM)

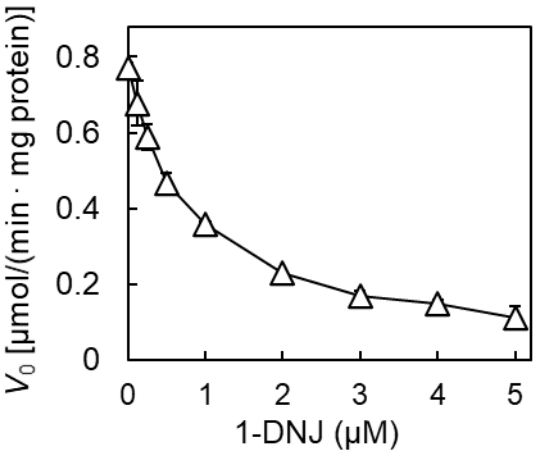
C


4
3
2
1
0
V_0 [μmol/(min · mg protein)]
0
20
40
60
80
100
Iminosugar (mM)

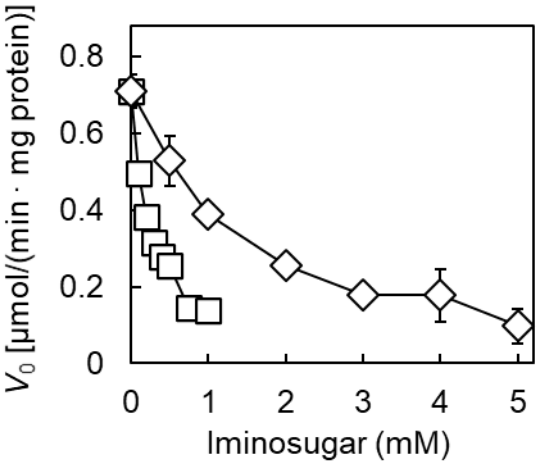
D


V_0 [μmol/(min · mg protein)]
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
Leaf extract (mg/mL)

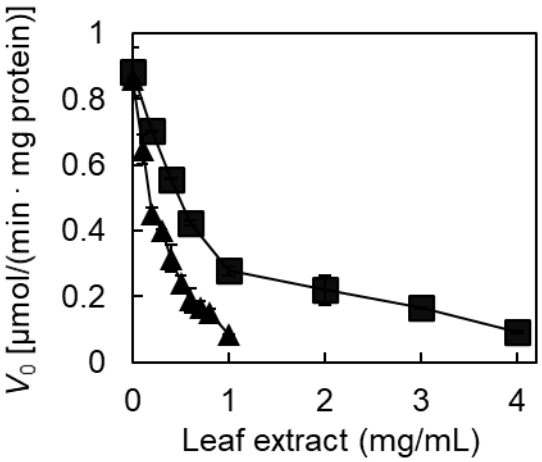
E


V_0 [μmol/(min · mg protein)]
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
1-DNJ (μM)

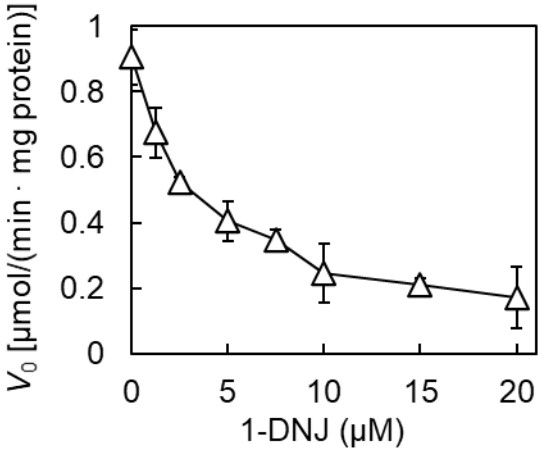
F


V_0 [μmol/(min · mg protein)]
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
Iminosugar (mM)

G


V_0 [μmol/(min · mg protein)]
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
Leaf extract (mg/mL)

H


V_0 [μmol/(min · mg protein)]
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
1-DNJ (μM)

I

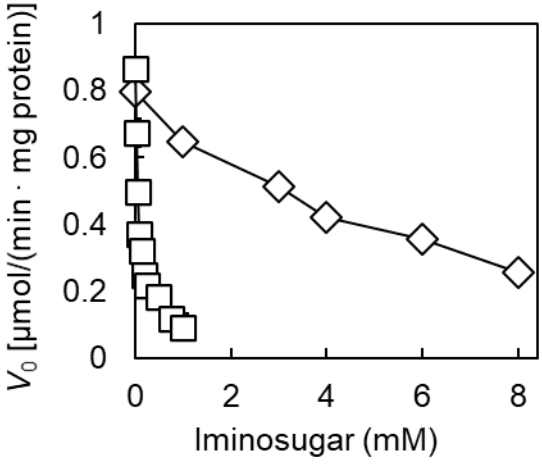

V_0 [μmol/(min · mg protein)]
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
Iminosugar (mM)

Fig. 3-2. Dependence on the extract and iminosugar concentrations of the reaction rate for the hydrolysis by rat intestinal acetone powder. The reaction was carried out with 0.10 mg protein/mL rat intestinal acetone powder solution, 15 mM G2-β-PNP (A–C), and 0.21 mg protein/mL rat intestinal acetone powder solution, 100 mM sucrose (D–F), or 50 mM isomaltose (G–I) in the presence of varying concentrations of powdered (closed triangle) or roasted leaf extract (closed square), 1-DNJ (open triangle), fagomine (open square), or GAL-DNJ (open diamond) at pH 6.0 at 37ºC. Error bars indicate SD values. The average of triplicate determination is shown.

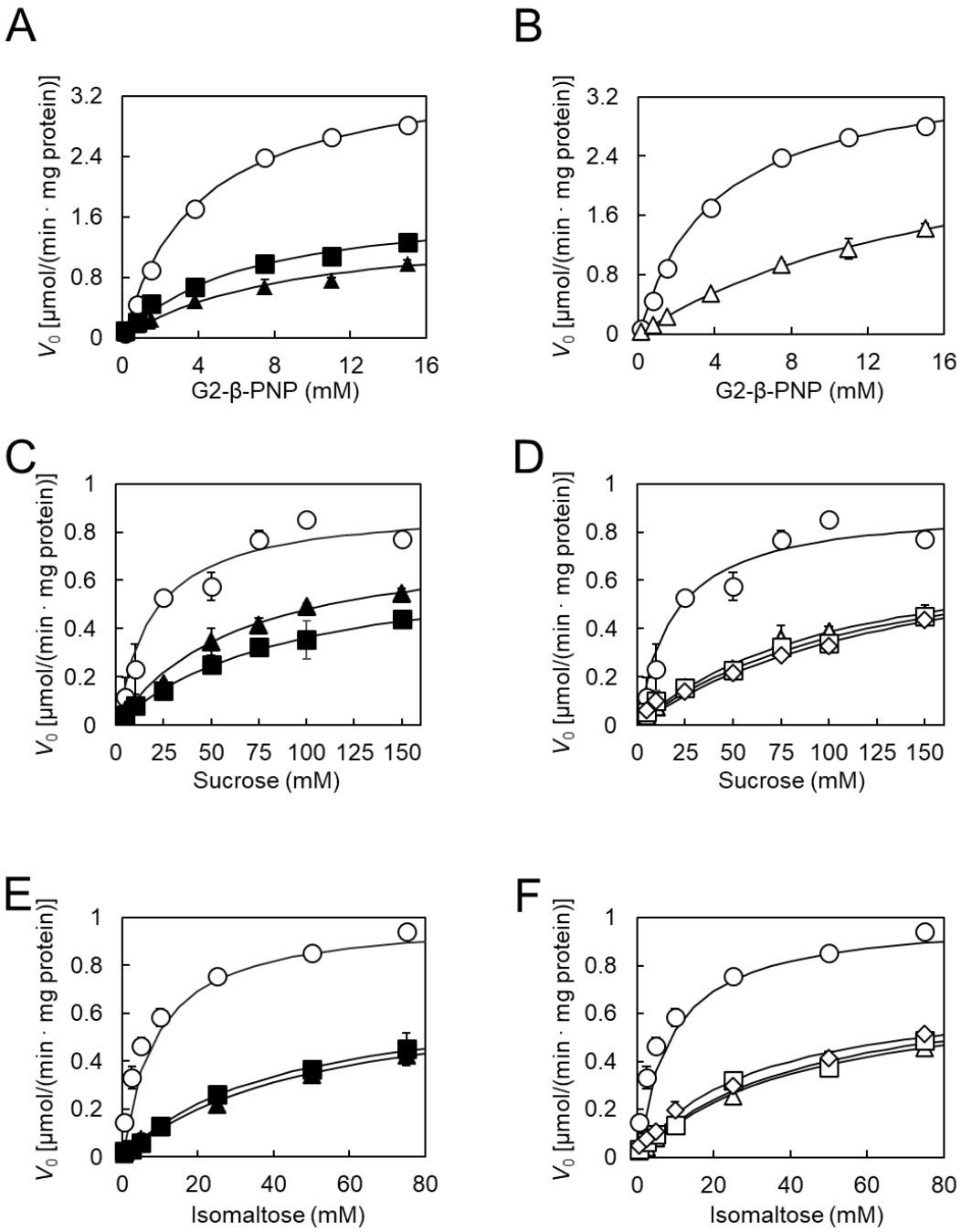

A
B
C
D
E
F
V_0 [μmol/(min · mg protein)]
G2-β-PNP (mM)
Sucrose (mM)
Isomaltose (mM)

Fig. 3-3. Dependence on the substrate concentration of the reaction rate for the hydrolysis by rat intestinal acetone powder in the presence of leaf extract or iminosugar. The reaction was carried out with 0.10 mg protein/mL rat intestinal acetone powder solution and varying concentrations of G2-β-PNP in the absence of iminosugar or extract (open circle) or in the presence of 4.0 mg/mL powdered leaf extract (closed triangle), 2.9 mg/mL of roasted leaf extracts (closed square), or 11 μg/mL 1-DNJ (open triangle) (A, B), 0.21 mg protein/ mL rat intestinal acetone powder solution and varying concentrations of sucrose in the absence of iminosugar or extract (open circle) or in the presence of 0.05 mg/mL powdered leaf extract (closed triangle), 0.15 mg/mL of roasted leaf extract (closed square), 0.14 μg/mL 1-DNJ (open triangle), 0.035 mg/mL fagomine (open square), or 0.41 mg/mL GAL-DNJ (open diamond) (C, D), or varying concentrations of isomaltose in the absence of iminosugar or extract (open circle) or in the presence of 0.24 mg/mL powdered leaf extract (closed triangle), 0.58 mg/mL of roasted leaf extract (closed square), 0.65 μg/mL 1-DNJ (open triangle), 0.011 mg/mL fagomine (open square), or 0.91 mg/mL GAL-DNJ (open diamond) (E, F) at pH 6.0 at 37ºC. Reaction rate (v_o) vs. substrate concentration is shown. Solid line represents the best fit to the experimental data using the Michaelis-Menten equation using Microsoft Excel. Error bars indicate SD values. The average of triplicate determination is shown.

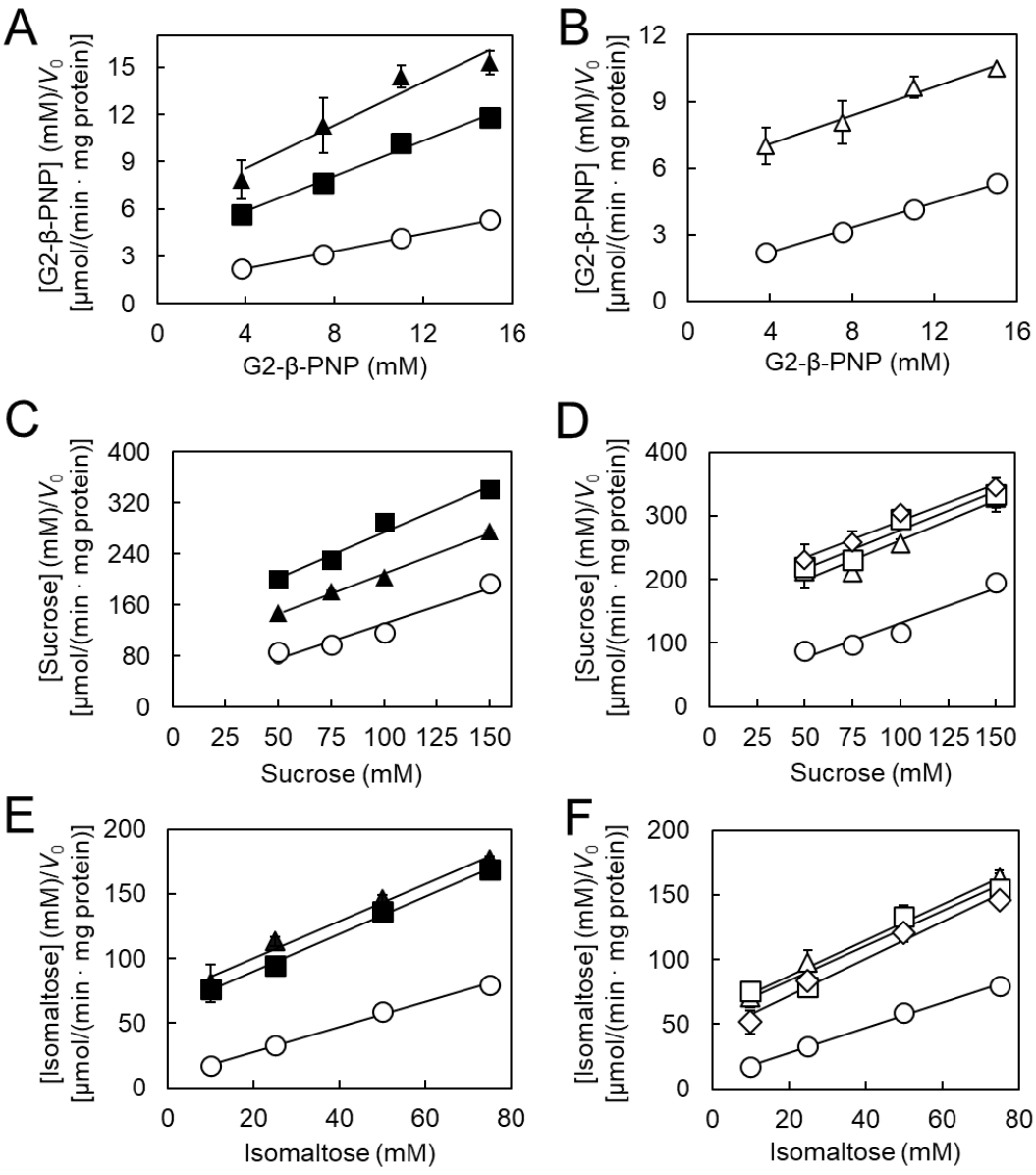


Fig. 3-4. Dependence on the substrate concentration of the reaction rate for the hydrolysis by rat intestinal acetone powder in the presence of leaf extract or iminosugar. The reaction conditions and symbols correspond to those of Fig. 3-3. Hanes-Woolf plot is shown.

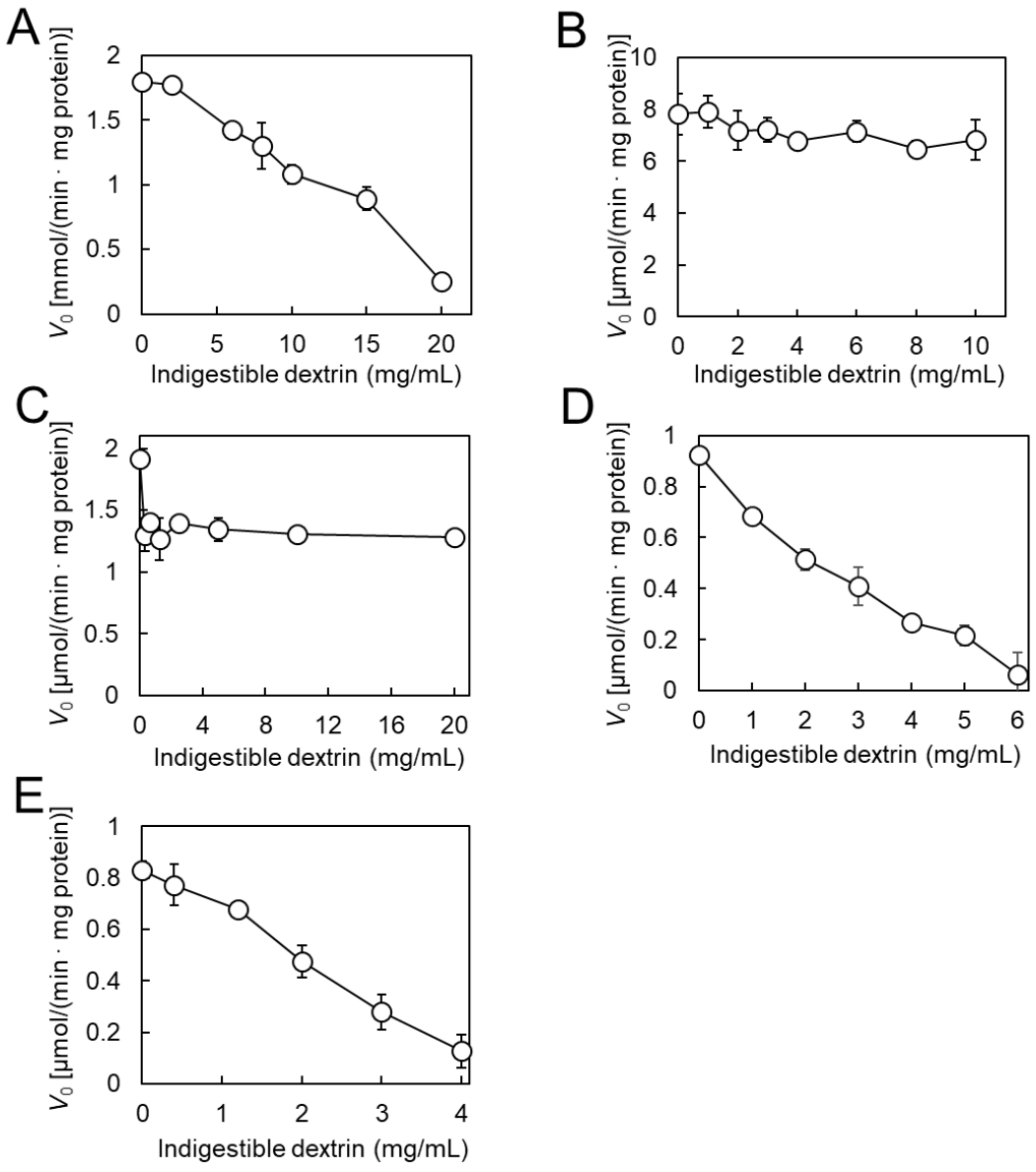


Fig. 3-5. Effects of the concentrations of indigestible dextrin on the hydrolysis by human α-amylase and that by rat intestinal acetone powder. (A) The reaction was carried out with 1.4 μg protein/mL human α-amylase and 20 mg/mL soluble starch at pH 6.0 at 37ºC. (B-E) The reaction was carried out with 0.10 mg protein/mL rat intestinal acetone powder solution with 50 mM maltose (B), 15 mM G2-β-PNP (C), and 0.21 mg protein/mL rat intestinal acetone powder solution with 100 mM sucrose (D), or 50 mM isomaltose (E) at pH 6.0 at 37ºC.

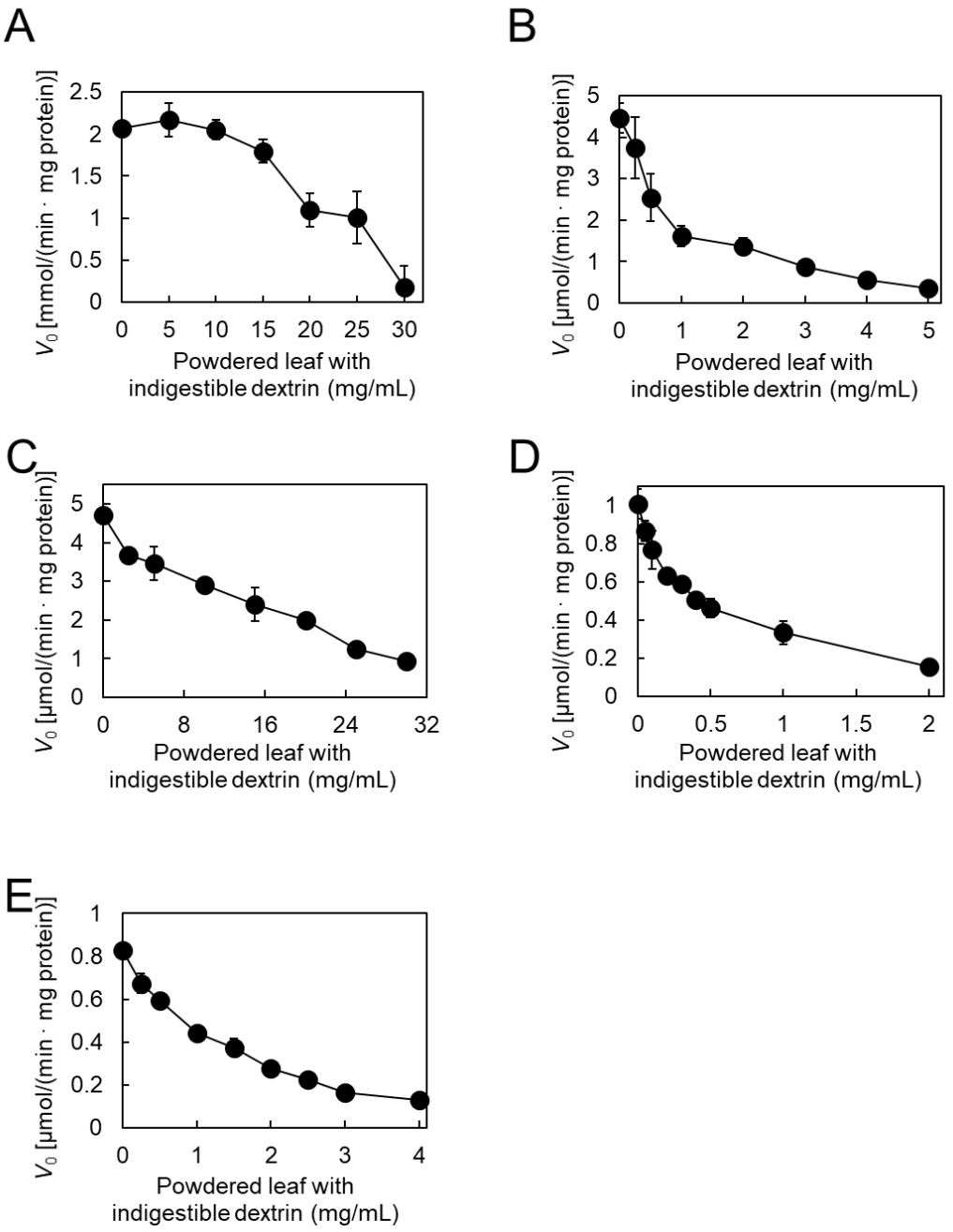


Fig. 3-6. Effects of the concentrations of powdered leaf extract blended with indigestible dextrin on the hydrolysis by human α-amylase and that by rat intestinal acetone powder. (A) The reaction was carried out with 1.4 μg protein/mL human α-amylase and 20 mg/mL soluble starch at pH 6.0 at 37ºC. (B-E) The reaction was carried out with 0.10 mg protein/mL rat intestinal acetone powder solution with 50 mM maltose (B), 15 mM G2-β-PNP (C), and 0.21 mg protein/mL rat intestinal acetone powder solution with 100 mM sucrose (D), or 50 mM isomaltose (E) at pH 6.0 at 37ºC.

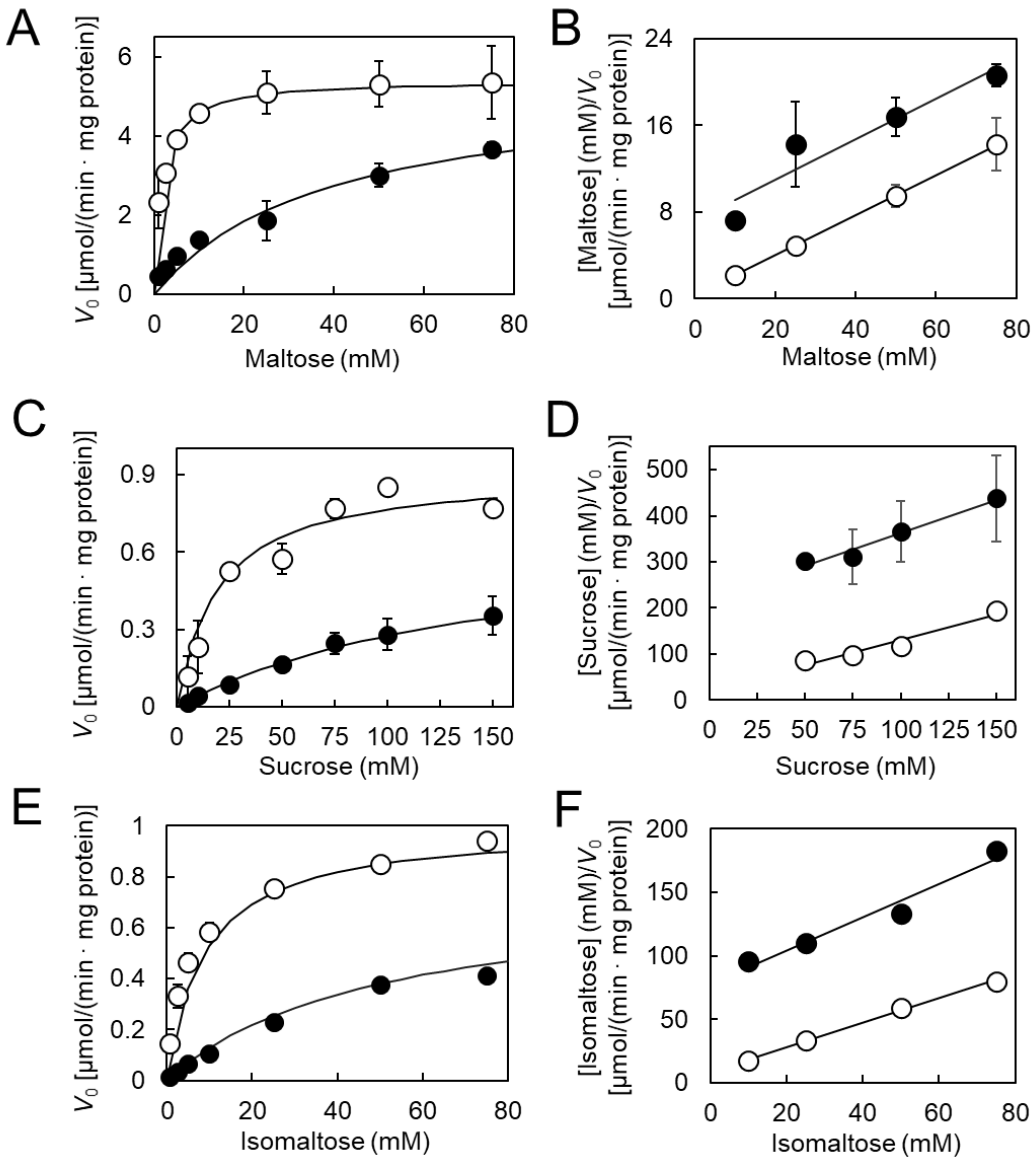


Fig. 3-7. Dependence on the substrate concentration of the reaction rate for the hydrolysis by rat intestinal acetone powder. The reaction was carried out with 0.10 mg protein/mL rat intestinal acetone powder solution in the absence (open circle) or in the presence (closed circle) of 0.65 mg/mL (A, B), 0.42 mg/mL (C, D), or 1.2 mg/mL (E, F) of powdered leaf extract blended with indigestible dextrin at pH 6.0 at 37ºC.

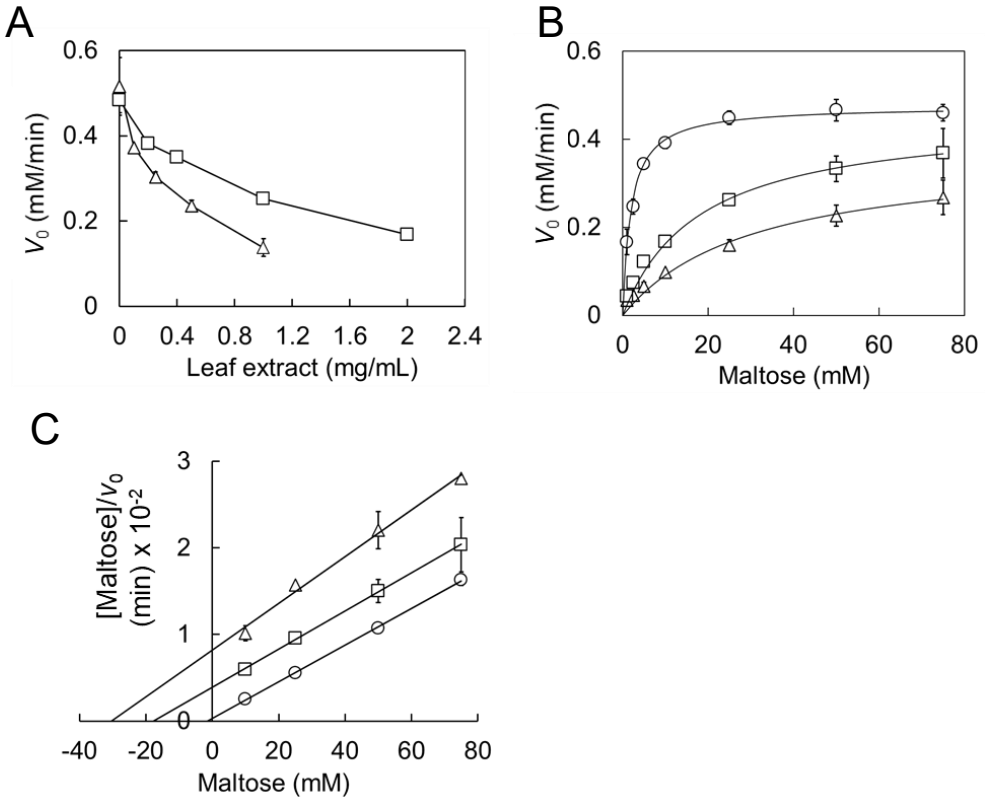


Fig. 3-8. Effects of the extracts of *M. australis* leaves on the hydrolysis of maltose by α-glucosidase. (A) Dependence on the concentration of leaf extracts on the reaction rate. The reaction was carried out with 50 mM maltose and varying concentrations of powdered (open triangle) or roasted (open square) leaf extracts at pH 6.0 at 37ºC. (B, C) Dependence on the substrate concentration of the reaction rate. The reaction was carried out with varying concentrations of maltose in the absence of iminosugar (open circle) or in the presence of 0.6 mg/mL powdered leaf extracts (open triangle) or roasted leaf extracts (open square) at pH 6.0 at 37ºC. (B) Reaction rate (v_o) *vs.* maltose concentration ([maltose]). Solid line represents the best fit to the experimental data using the Michaelis-Menten equation using Microsoft Excel. (C) Hanes-Woolf plot ([maltose] /v_o *vs.* [maltose]). The K_m and V_{max} values were calculated to be 1.9 mM and 0.48 mM/min, respectively, in the absence of leaf extracts, and the K_{mapp} and V_{max} values were calculated to be 30 mM and 0.37 mM/min, respectively, in the presence of 0.6 mg/mL of powdered leaf extract, and 18 mM and 0.45 mM/min, respectively, in the presence of 0.6 mg/mL of roasted leaf extract. Error bars indicate SD values. The average of triplicate determination is shown.

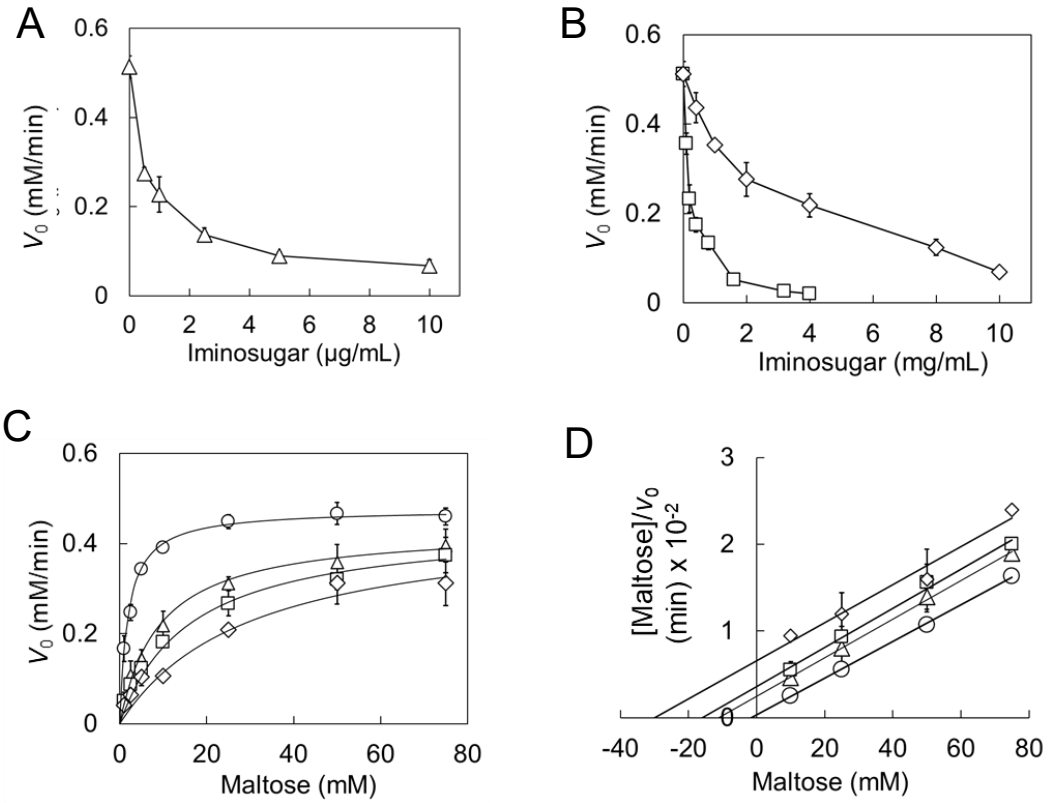


Fig. 3-9. Effects of 1-DNJ, fagomine, and GAL-DNJ on the hydrolysis of maltose by α-glucosidase. (A, B) Dependence on the concentration of 1-DNJ, fagomine, and GAL-DNJ on the reaction rate. The reaction was carried out with 50 mM maltose and varying concentrations of 1-DNJ (open triangle) (A), fagomine (open square) (B), or GAL-DNJ (open diamond) (B) at pH 6.0 at 37ºC. (C, D) Dependence on the substrate concentration of the reaction rate. The reaction was carried out with varying concentrations of maltose in the absence of iminosugar (open circle) or in the presence of 0.25 μg/mL of 1-DNJ (closed triangle), 0.1 mg/mL of fagomine (closed square), or 1.5 mg/mL of GAL-DNJ (closed diamond) at pH 6.0 at 37ºC. (D) v_o vs. [maltose]. Solid line represents the best fit to the experimental data using the Michaelis-Menten equation using Microsoft Excel. (D) Hanes-Woolf plot ([maltose] /v_o vs. [maltose]). The K_m and V_{max} values were calculated to be 1.9 mM and 0.48 mM/min, respectively, in the absence of iminosugar, and the K_{mapp} and V_{max} values were calculated to be 9.0 mM and 0.44 mM/min, respectively, in the presence of 0.25 μg/mL of 1-DNJ, 16 mM and 0.44 mM/min, respectively, in the presence of 0.1 mg/mL of fagomine, and 30 mM and 0.46 mM/min, respectively, in the presence of 1.5 mg/mL of GAL-DNJ. Error bars indicate SD values. The average of triplicate determination is shown.

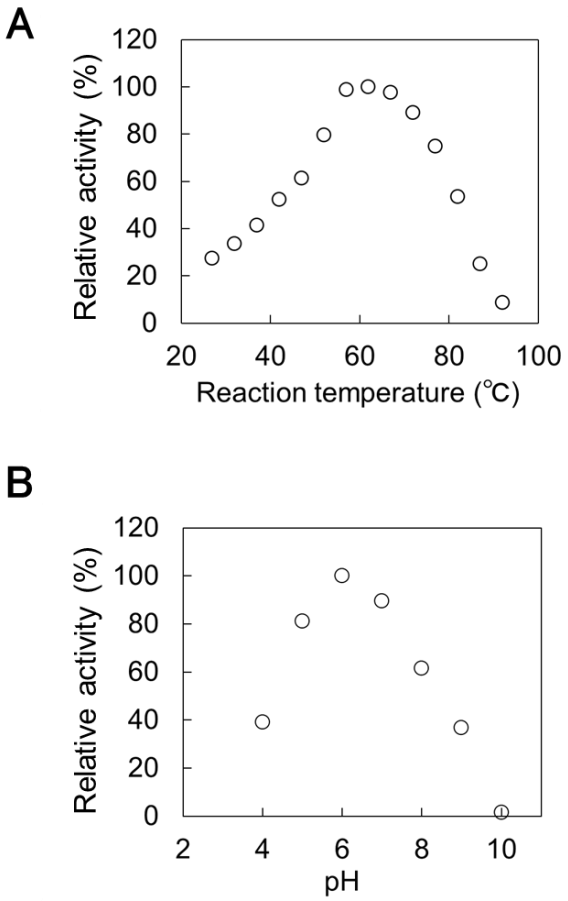


Fig. 3-10. Effects of reaction temperature and pH on the α-glucosidase activity. (A) Temperature dependence. The activity at 62ºC was defined as 100%. The reaction was carried out with 3.5 mg/mL extract from rat intestinal acetone powder, 50 mM maltose at pH 6 at 27–92ºC. (B) pH dependence. The activity at pH 6 was defined as 100%. The reaction was carried out with 3.5 mg/mL extract, 50 mM maltose at pH 4–6 at 37ºC.

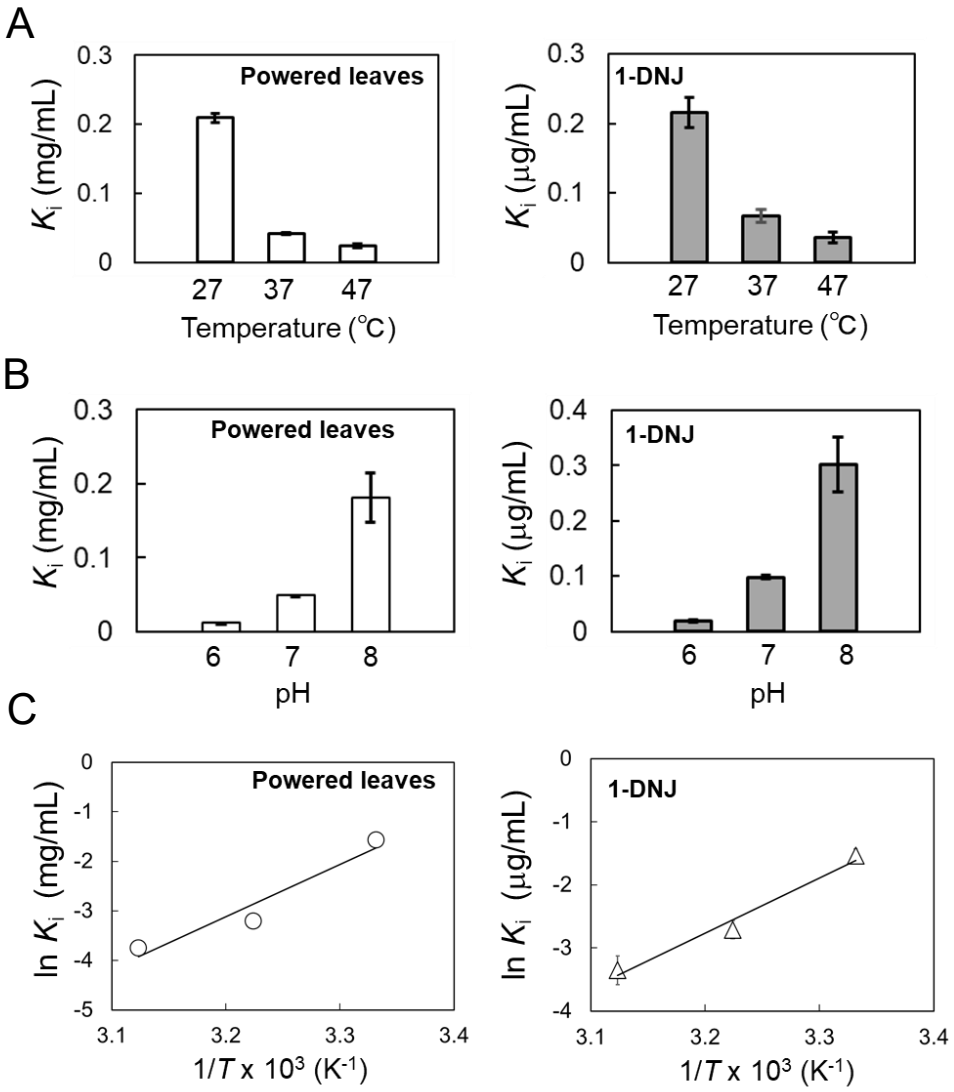


Fig. 3-11. Effects of reaction temperature and pH on the inhibitory effects of the extracts of *M. australis* powdered leaves and 1-DNJ toward the hydrolysis of maltose by α-glucosidase. Notes: (A) Dependence of K_i values at pH 6.0 on reaction temperature. The K_i values at 27ºC, 37ºC, and 47ºC are 0.21 ± 0.01, 0.040 ± 0.002, and 0.024 ± 0.003 mg/mL for the extracts of powdered leaf and 0.22 ± 0.02, 0.067 ± 0.001, and 0.035 ± 0.008 g/mL for 1-DNJ. (B) Dependence of K_i values at 37ºC on pH. The K_i values at pH 6, 7, and 8 are 0.04 ± 0.01, 0.049 ± 0.001, and 0.18 ± 0.01 mg/mL for the extracts of powdered leaves and 0.067 ± 0.001, 0.098 ± 0.003, and 0.30 ± 0.05 ± mg/mL for 1-DNJ. (C) van't Hoff plot of K_i values. K_i values were plotted against the reciprocal of the absolute temperature. Enthalpy changes (ΔH^o) were calculated from the slope to be -87.6 ± 6.4 kJ mol^{-1} for the extracts of powdered leaves and -72.8 ± 12.9 kJ mol^{-1} for 1-DNJ. Error bars indicate SD values. The average of triplicate determination is shown.

Chapter 4

Quantification of iminosugar, anthocyanin, and glucose in *Morus australis* fruit extract*

Introduction

Of various parts of the mulberry tree, the leaf and fruit offer the health benefits. Consumption of the leaf tea lowered the postprandial increase in blood glucose concentration in human (18), which contributes to glycemic control. This effect is due to inhibition of α-glucosidase by iminosugar, especially 1-deoxynojirimycin (1-DNJ). In mammals, α-glucosidase exists as mucosal brush border-anchored maltase-glucoamylase (53) and sucrase-isomaltase complexes (54). Consumption of the fruit prevented diabetes and diabetic dementia in mice (27). This effect seemed to be due to certain bioactive components that increased the expression level of brain-derived neurotrophic factor (BDNF) in brain (55). 1-DNJ inhibit α-amylase weekly. In contrast, azadirachtins from *Azadirachta indica*, 4-hydroxyisoleucine from *Trigonella foenum-graecum,* and cinnamaldehyde from *Cinnamomum sieboldii* have been reported to have α-amylase and α-glucosidase inhibition activities (56). *Azadirachta indica*, *Trigonella foenum-graecum,* and *Cinnamomum sieboldii* are used in medicinal plants for the management of diabetes

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*The content described in this Chapter was originally published in the following paper.
Qiao, Y., Ikeda, Y., Ito, M., Kimura, T., Ikeuchi, T., Takita, T., and Yasukawa, K. (2022) Inhibition of α-amylase and α-glucosidase by *Morus australis* fruit extract and its components iminosugar, anthocyanin, and glucose. *J. Food Sci.*, in press

although the clinical effectiveness remains controversial (56).

In this study, we quantified these components in *M. australis* fruit extract and discuss which components in *M. australis* fruit extract are involved in the inhibition of α-glucosidase.

Materials and Methods

Materials – *M. australis* fruits were purchased from Urasoe-shi silver human resources center. 1-DNJ was purchased from Fujifilm Wako Pure Chemical. Fagomine and GAL-DNJ were purified from mulberry leaves as reported previously (24). Cyanidin-3-glucoside (C3G) and cyanidin-3-rutinoside (C3R) were purchased from Nagara Science (Gifu, Japan).

Preparation of M. australis fruit extracts – Names and characteristics of four subspecies (No. 1–4) of *M. australis* fruit were as follows. No. 1, MiyaT-17, tetraploid derived from No. 2; No. 2, Miya-17, diploid collected in Miyakojima, Okinawa; No. 3, IkeT-17, tetraploid derived from No. 4; No. 4, Ike-17, diploid collected in Ikemajima, Okinawa. In our laboratory, these four fruits were powdered by freeze-drying and grinding in a mortar. Boiled water (25 mL) was added to the powder (2.5 g) and stirred for 10 min. The solution was suction-filtrated using 0.7 μm cellulose filter paper (Middlesex). The filtrate was collected and used as the fruit extract.

Liquid chromatography-MS/MS (LC-MS/MS) analysis – LC-MS/MS analysis was carried out almost the same as Chapter 2. Briefly, powdered *M. australis* fruits (500 mg) were dissolved in 50% ethanol (5.0 mL) followed by sonication for 5 min and centrifugation (10,000 x *g*, 4ºC) for 5 min. The supernatant was filtered with 0.4 μm pore

size filter (Sartorius Stedim Biotech), diluted with 0.1% formic acid, 50% acetonitrile to be 1% v/v for the quantification of 1-DNJ and GAL-DNJ and to be 10% for the quantification of fagomine. Standard 1-DNJ, fagomine, and GAL-DNJ were dissolved in 0.1% formic acid, 50% acetonitrile to be 200–1,000 ng/mL. The filtrates were subjected to LC-MS/MS analysis using a TSK gel Amide-80 (particle size 5 μm; 100 mm × 2.0 mm i.d., Tosoh) on a LC-20A system (Shimadzu) connected to LCMS-8045 (Shimadzu). The mobile phase consisted of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B). Elution gradient were carried out 20%–60% solvent B for 0–2.0 min, 60% solvent B for 2.0–5.5 min, 60%–20% solvent B for 5.5–5.6 min, and 20% solvent B for 5.6–8.0 min at a flow rate of 0.2 mL/min. Other conditions corresponded to those described in Chapter 2. To estimate the concentrations of 1-DNJ, fagomine, and GAL-DNJ in powdered *M. australis* fruit extracts, each iminosugar was used to make a standard curve.

HPLC analysis – HPLC analysis was performed according to Yu et al. (58). Briefly, boiled water (50 mL) was added to the powder of *M. australis* fruit (2 g) and stirred for 10 min. The solution was suction-filtrated. The filtrate was separated and filtered (0.4 μm pore size; Sartorius Stedim Biotech). The filtrate was diluted with ethanol containing 1% v/v HCl. Conditions of HPLC are as follows: apparatus, eAlliance2695 (Waters, Milford, MA); column, Capcell Pak C18 (250 mm × 4.6 mm i.d., Shiseido, Tokyo, Japan); column oven temperature, 25ºC; mobile phase, 0.1% w/v phosphate buffer (pH 2.0) (A) and acetonitrile (B); mobile phase flow rate, 1.0 mL/min; injection volume, 10 μL. Elution gradients are as follows: 0–7 min, 6%–9% solvent B; 7–18 min, 9%–11% solvent B; 18–30 min, 11%–30% solvent B; 30–34 min, 30%–15% solvent B; and 34–50 min, 15% solvent B. To estimate the concentrations of C3G and C3R in powdered *M. australis* fruit

extracts, standard C3G or C3R (Fig. 4-1) was used to make a standard curve.

Quantification of glucose in M. australis fruit – Quantification of glucose in the fruit extract using Glucose CII test (Fujifilm Wako Pure Chemical) method.

Results and Discussion

Quantification of iminosugars in M. australis fruits –In this study, to quantify iminosugars in *M. australis* fruit, each of standard 1-DNJ, fagomine, and GAL-DNJ were applied to LC-MS/MS to make a calibration curve. Figures 4-2 and 4-3 show the total ion scanning profile and MRM chromatogram of the total ions, respectively, of each iminosugar. Figure 4-4 shows the calibration curve, showing that the peak intensity increased linearly with increasing the concentration (0–1,000 ng/mL) of each iminosugar. Four fruit extracts (No. 1–4) were applied to LC-MS/MS. The MRM chromatogram showed one peak at 5.4 min for 1-DNJ (Fig. 4-5A, D, G, and J), one peak at 2.7 min for fagomine (Fig. 4-5B, E, H, and K), and one peak at 4.3 min for GAL-DNJ (Fig. 4-5C, F, I, and L). Based on the calibration curves (Fig. 4-4), the concentrations of iminosugar in the dried *M. australis* fruit were 0.04–0.29 mg/g for 1-DNJ, 0.10–0.37 mg/g for fagomine, and 0.49–0.78 mg/g for GAL-DNJ (Table 1). When the concentration of 1-DNJ in the powdered *M. australis* leaf was set 100%, those in *M. australis* fruit were 1.0–7.3%.

Besides *Morus* fruit, sweet cheery contains various bioactive compounds and has great attention as a healthy sweetener (59). Gonçalves et al. identified 23 phenolic compounds in five varieties of sweet cherries (59). In that study, they showed that the IC_{50} value of the most active sweet cheery extract for α-glucosidase was 0.013 mg/mL, which corresponded to 0.42 mg/mL when the average yield after drying (3.1%) was considered (60) and was comparable to the IC_{50} values of *M. australis* fruit extract (0.13–

8.6 mg/mL) obtained in this study (Table 1).

Quantification of anthocyanin in M. australis fruit – Figure 4-6A and B show the elution pattern of C3G and C3R, respectively, in the reversed-phase HPLC. C3G and C3R exhibited a single peak at 25.90 and 26.96 min, respectively. Figure 4-7 shows the calibration curve, showing that the peak intensity increased linearly with increasing concentration of C3G or C3R. Figure 4-6C–G show the HPLC patterns of the fruit extract No. 1–4, and the powdered leaf extract, respectively. No. 1 and 2 exhibited the peak corresponding to C3G and C3R (Fig. 4-6C, D) while No. 3 or 4 or the powdered leaf extract did not (Fig. 4-6E–G). Based on the calibration curves (Fig. 4-7), the concentrations of C3G and C3R were 12 and 3.9 mg/g dried fruit, respectively, for No. 1 and 4.4 and 1.2 mg/g dried fruit, respectively, for No. 2 (Table 1). These results suggested that the concentration of anthocyanin in *M. australis* fruit depends on strains considerably.

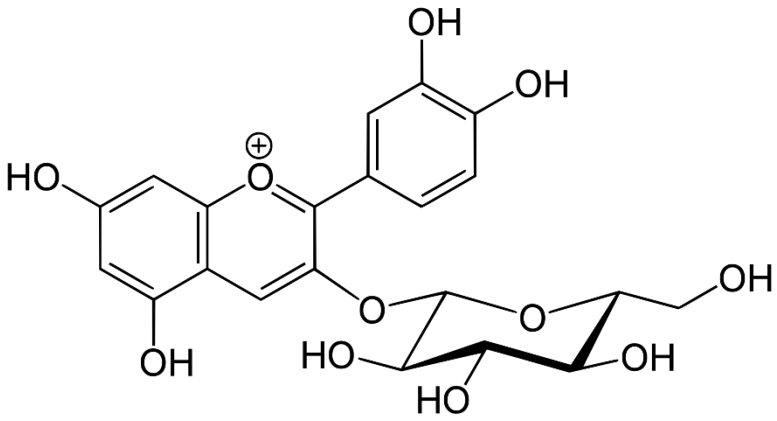
Conclusion – Our results indicated that, 1-DNJ, anthocyanin, and glucose are involved in *M. australis* fruit. This is in contrast to that in *M. australis* leaf, 1-DNJ is a main inhibitor of α-glucosidase, and neither anthocyanin nor glucose are contained.

Table 1. Concentrations of iminosugar, anthocyanin, and glucose in the fruit and powdered leaf extracts.

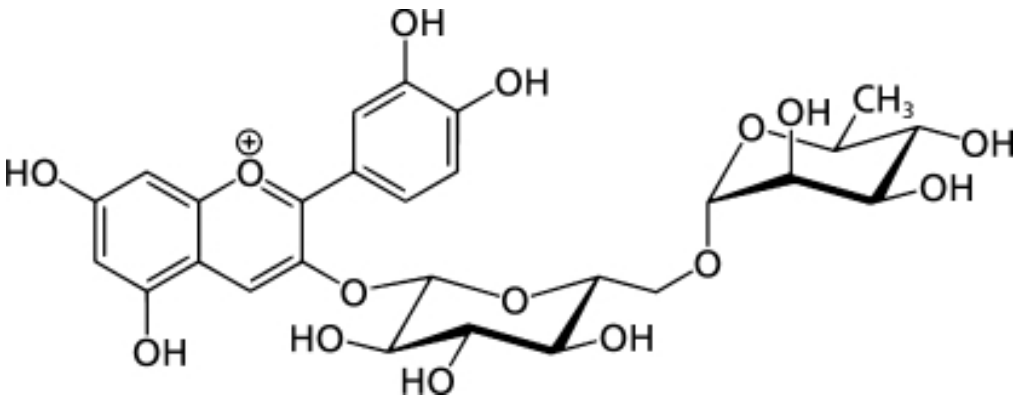
	1-DNJ (mg/g)	Fagomine (mg/g)	GAL-DNJ (mg/g)	C3G (mg/g)	C3R (mg/g)	Glucose (mg/g)
Fruit extract #1	0.17 (4.2%)[a]	0.37 (80%)	0.65 (26%)	12	3.9	262
Fruit extract #2	0.29 (7.3%)	0.17 (37%)	0.78 (31%)	4.4	1.2	123
Fruit extract #3	0.04 (1.0%)	0.13 (28%)	0.49 (20%)	—	—	260
Fruit extract #4	0.18 (4.5%)	0.10 (22%)	0.52 (21%)	—	—	211
Powdered leaf extract	4.0 (100%)	0.46 (100%)	2.5 (100%)	0	0	0

Values for the concentration of 1-DNJ, fagomine, and GAL-DNJ of powdered leaf extract are from Chapter 2.

[a]Values in parenthesis indicate relative values (%) compared to powdered leaf extract.



Cyanidin-3-glucoside



Cyanidin-3-rutinoside

Fig. 4-1. Structures of cyanidin-3-glucoside and cyanidin-3-rutinoside.

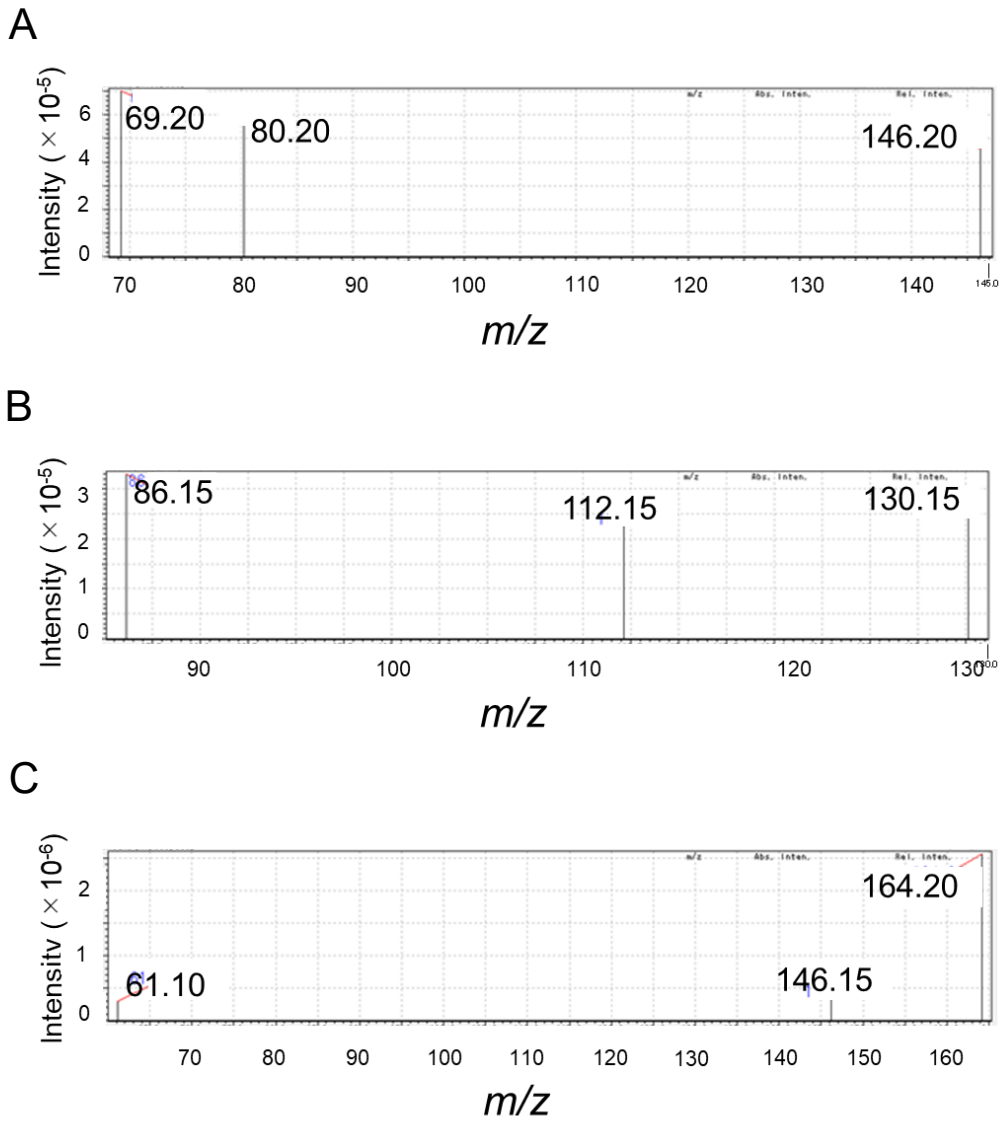


Fig. 4-2. Product ion spectra of 1-DNJ, fagomine, and GAL-DNJ. Standard 1-DNJ (5 μL in 1.0 μg/mL) (A), fagomine (5 μL in 1.0 μg/mL) (B), GAL-DNJ (5 μL in 1.0 μg/mL) (C) were applied. Product ion spectra of the ions at *m/z* 164.20 $[M+H]^+$ for 1-DNJ (A), at *m/z* 148.20 $[M+H]^+$ for fagomine (B), and at *m/z* 326.20 $[M+H]^+$ for GAL-DNJ (C) are shown.

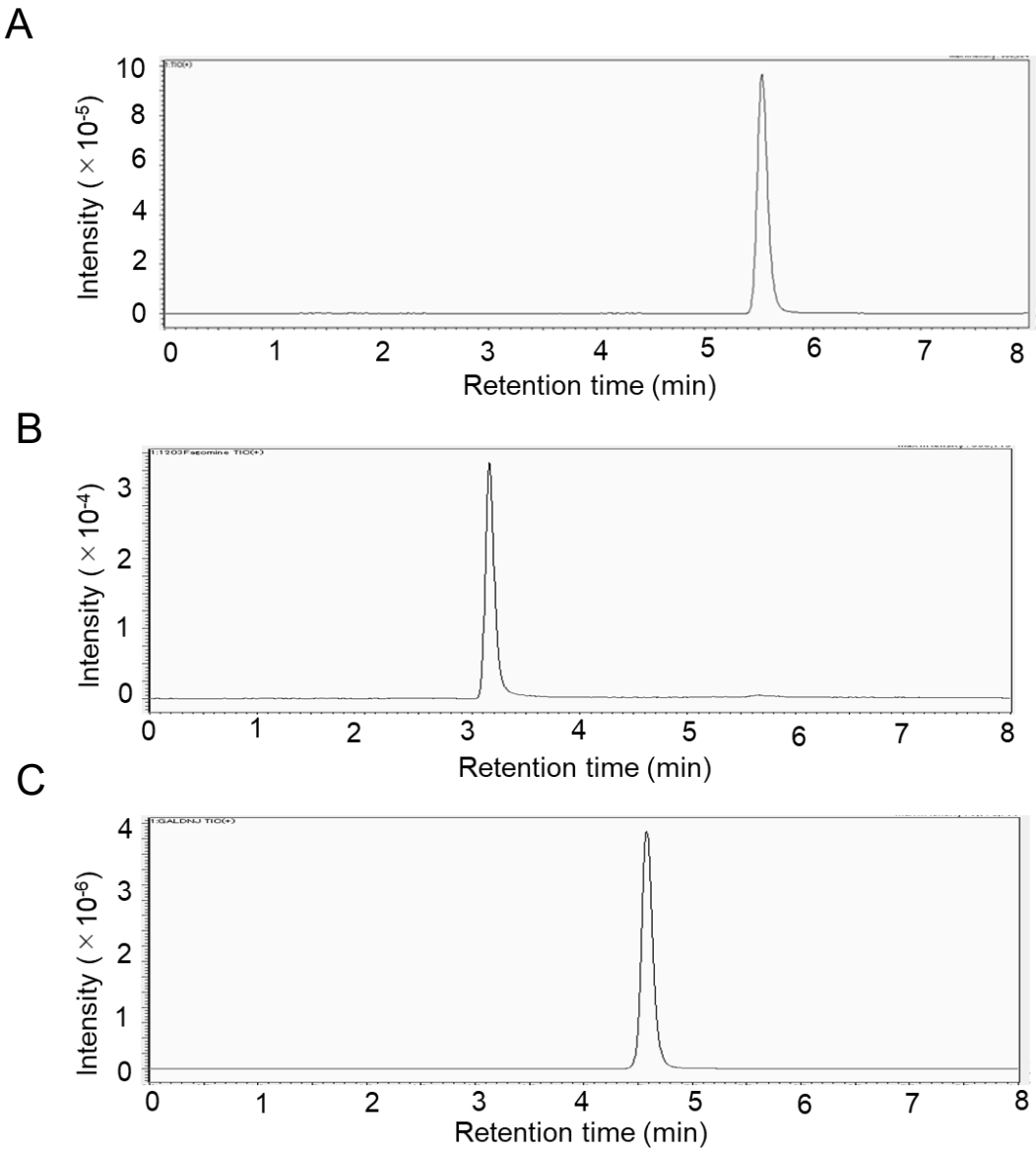


Fig. 4-3. MRM chromatogram of 1-DNJ, fagomine, and GAL-DNJ. Standard 1-DNJ (5 μL in 1.0 μg/mL) (A), fagomine (5 μL in 1.0 μg/mL) (B), GAL-DNJ (5 μL in 1.0 μg/mL) (C) were applied. MRM chromatogram of the total of ions at *m/z* 69.20 $[M+H-95]^+$, 80.20 $[M+H-84]^+$, and 146.20 $[M+H-H_2O]^+$ for 1-DNJ (A), at 86.15 $[M+H-62]^+$, 112.15 $[M+H-2H_2O]^+$, and 130.15 $[M+H-H_2O]^+$ for fagomine (B), and at 61.10 $[M+H-265]^+$, 146.15 $[M+H-180]^+$, and 164.20 $[M+H-162]^+$ for GAL-DNJ (C) are shown.

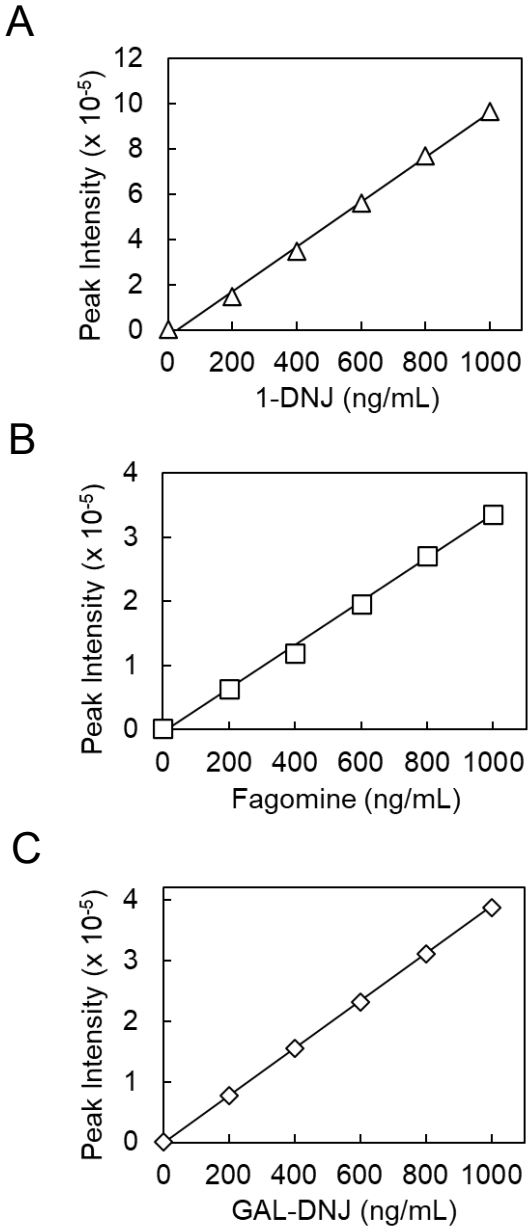


Fig. 4-4. Calibration curves of 1-DNJ, fagomine, and GAL-DNJ. (A) 1-DNJ (0–1,000 ng/mL). (B) Fagomine (0–1,000 ng/mL). (C) GAL-DNJ (0–1,000 ng/mL).

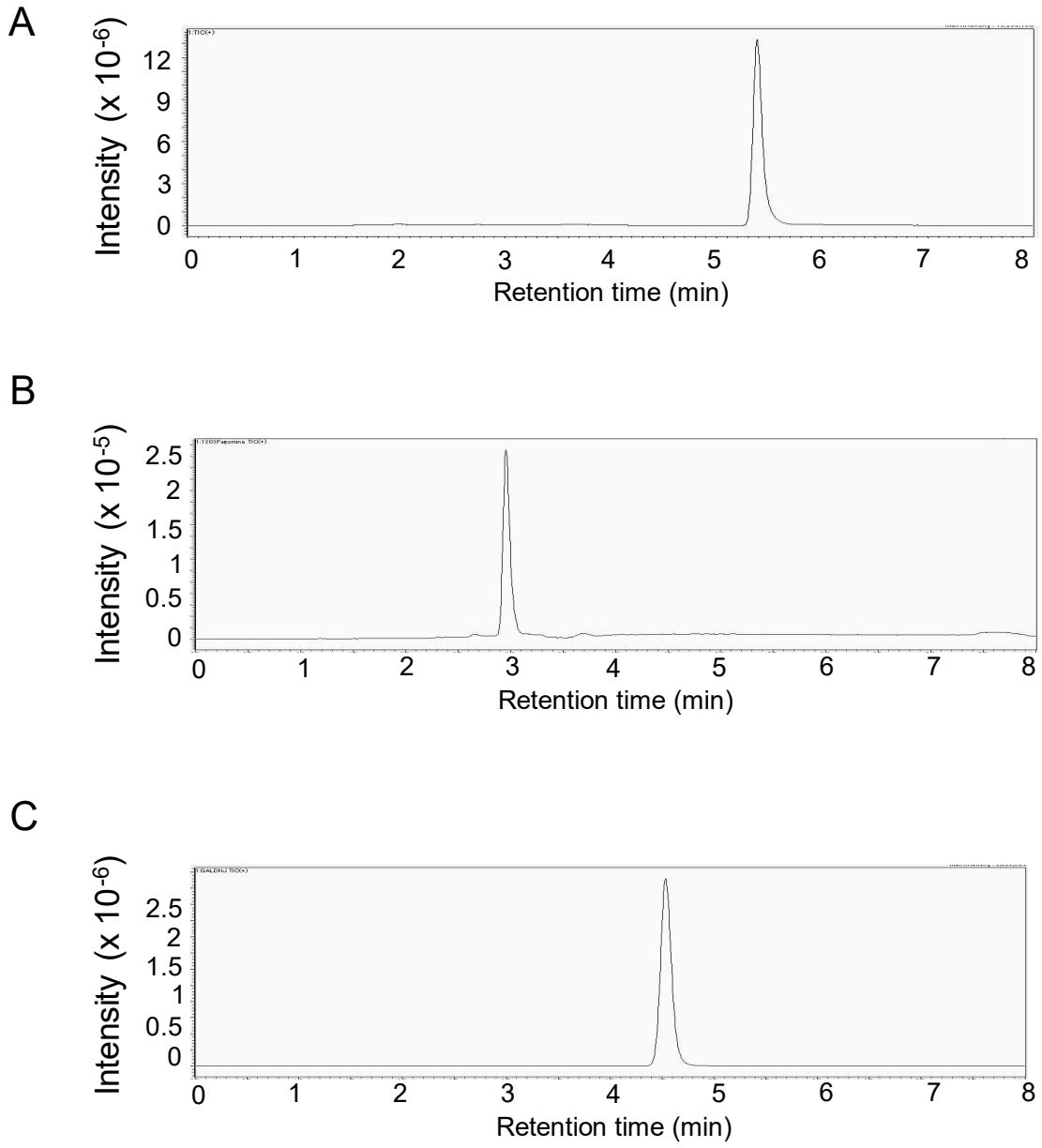


Fig. 4-5-1. MRM chromatogram of *M. australis* fruit extract. The extracts of *M. australis* fruit No. 1 (A–C) were applied. MRM chromatogram of the total of ions at m/z 69.20 $[M+H-95]^+$, 80.20 $[M+H-84]^+$, and 146.20 $[M+H-H_2O]^+$ for 1-DNJ (A), at 86.15 $[M+H-62]^+$, 112.15 $[M+H-2H_2O]^+$, and 130.15 $[M+H-H_2O]^+$ for fagomine (B), and at 61.10 $[M+H-265]^+$, 146.15 $[M+H-180]^+$, and 164.20 $[M+H-162]^+$ for GAL-DNJ (C) are shown.

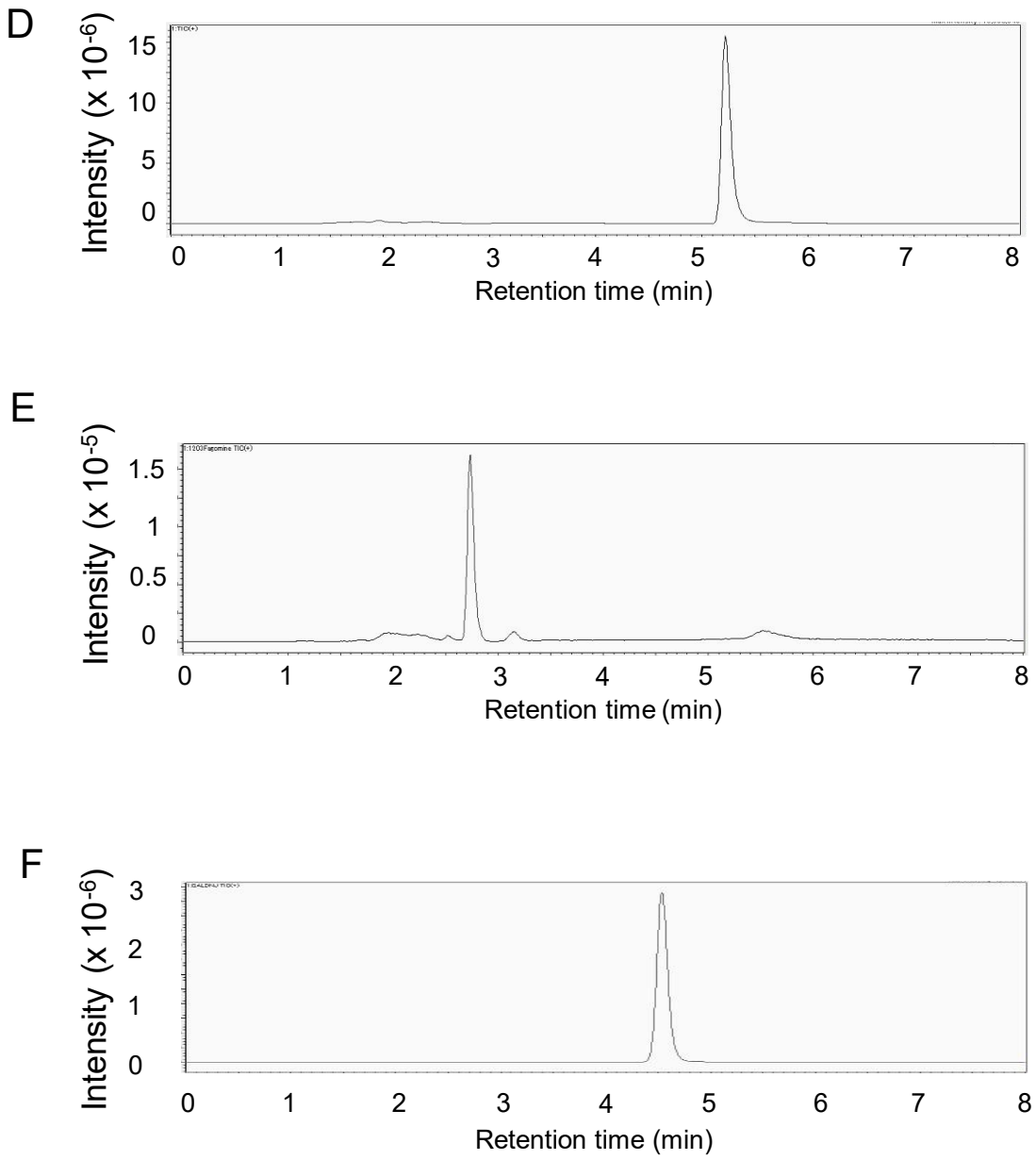


Fig. 4-5-2. MRM chromatogram of *M. australis* fruit extract. The extracts of *M. australis* fruit No. 2 (D–F) were applied. MRM chromatogram of the total of ions at m/z 69.20 $[M+H-95]^+$, 80.20 $[M+H-84]^+$, and 146.20 $[M+H-H_2O]^+$ for 1-DNJ (D), at 86.15 $[M+H-62]^+$, 112.15 $[M+H-2H_2O]^+$, and 130.15 $[M+H-H_2O]^+$ for fagomine (E), and at 61.10 $[M+H-265]^+$, 146.15 $[M+H-180]^+$, and 164.20 $[M+H-162]^+$ for GAL-DNJ (F) are shown.

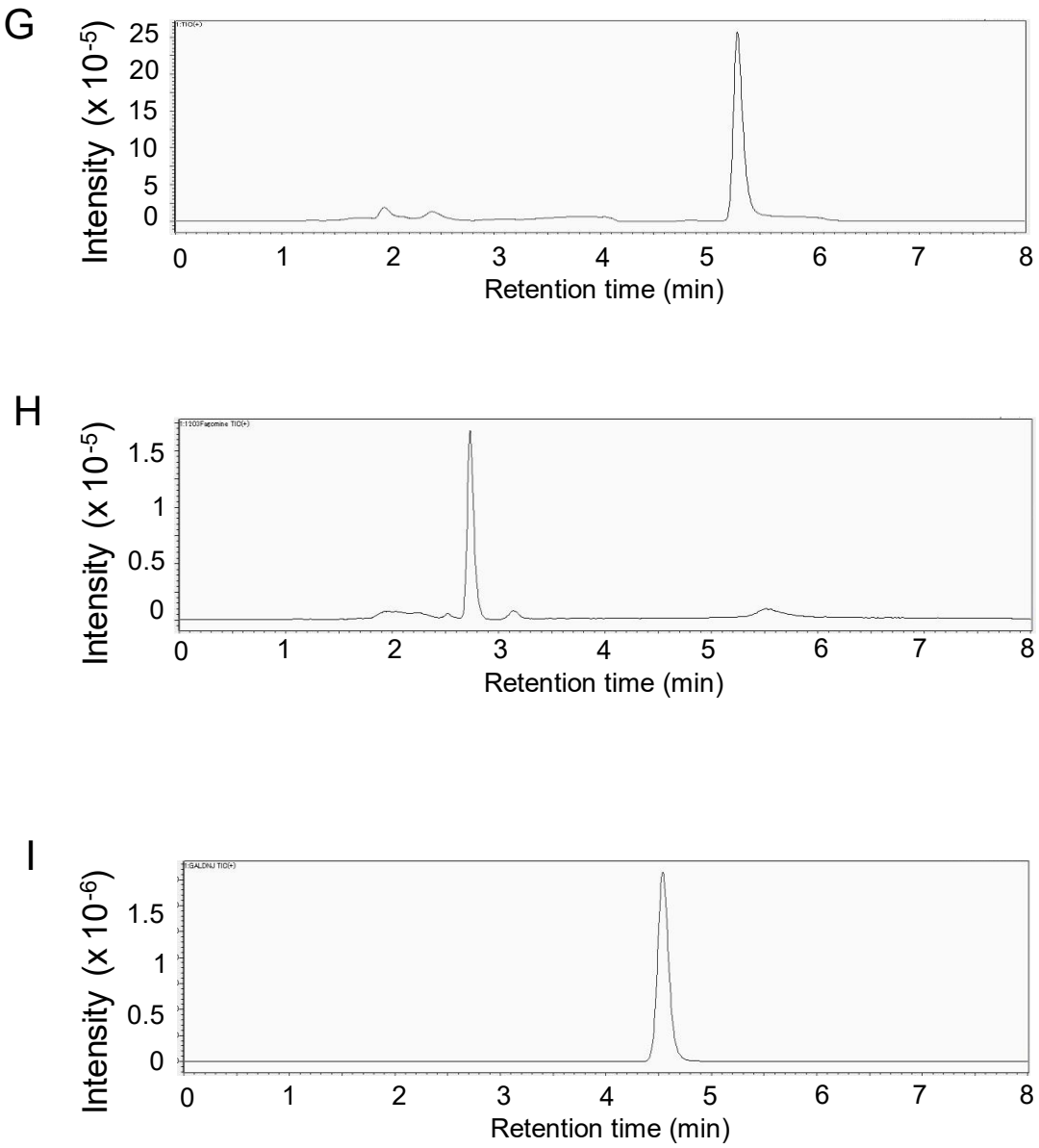


Fig. 4-5-3. MRM chromatogram of *M. australis* fruit extract. The extracts of *M. australis* fruit No. 3 (G–I) were applied. MRM chromatogram of the total of ions at m/z 69.20 $[M+H-95]^+$, 80.20 $[M+H-84]^+$, and 146.20 $[M+H-H_2O]^+$ for 1-DNJ (G), at 86.15 $[M+H-62]^+$, 112.15 $[M+H-2H_2O]^+$, and 130.15 $[M+H-H_2O]^+$ for fagomine (H), and at 61.10 $[M+H-265]^+$, 146.15 $[M+H-180]^+$, and 164.20 $[M+H-162]^+$ for GAL-DNJ (I) are shown.

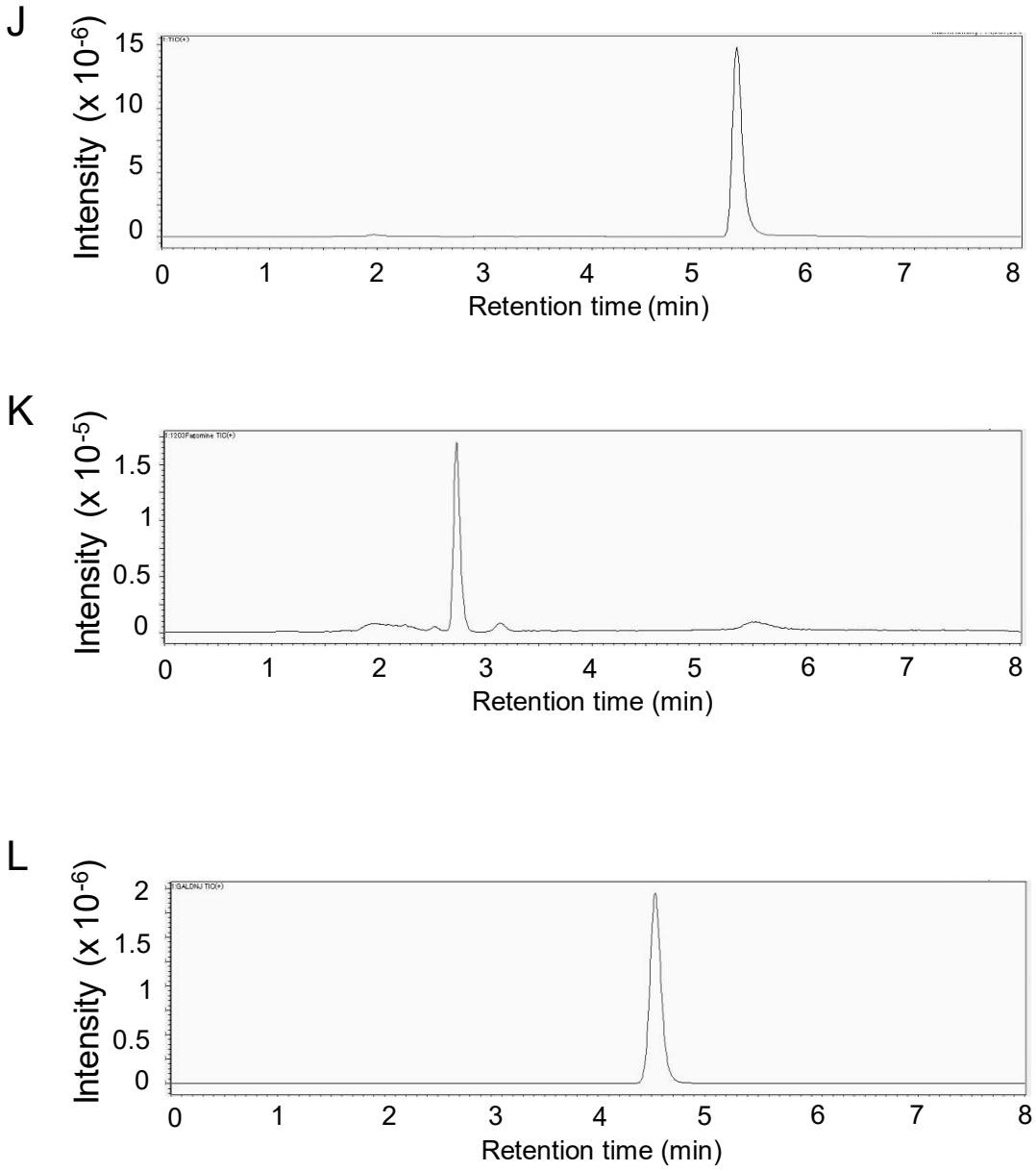


Fig. 4-5-4. MRM chromatogram of *M. australis* fruit extract. The extracts of *M. australis* fruit No. 4 (J–L) were applied. MRM chromatogram of the total of ions at m/z 69.20 $[M+H-95]^+$, 80.20 $[M+H-84]^+$, and 146.20 $[M+H-H_2O]^+$ for 1-DNJ (J), at 86.15 $[M+H-62]^+$, 112.15 $[M+H-2H_2O]^+$, and 130.15 $[M+H-H_2O]^+$ for fagomine (K), and at 61.10 $[M+H-265]^+$, 146.15 $[M+H-180]^+$, and 164.20 $[M+H-162]^+$ for GAL-DNJ (L) are shown.

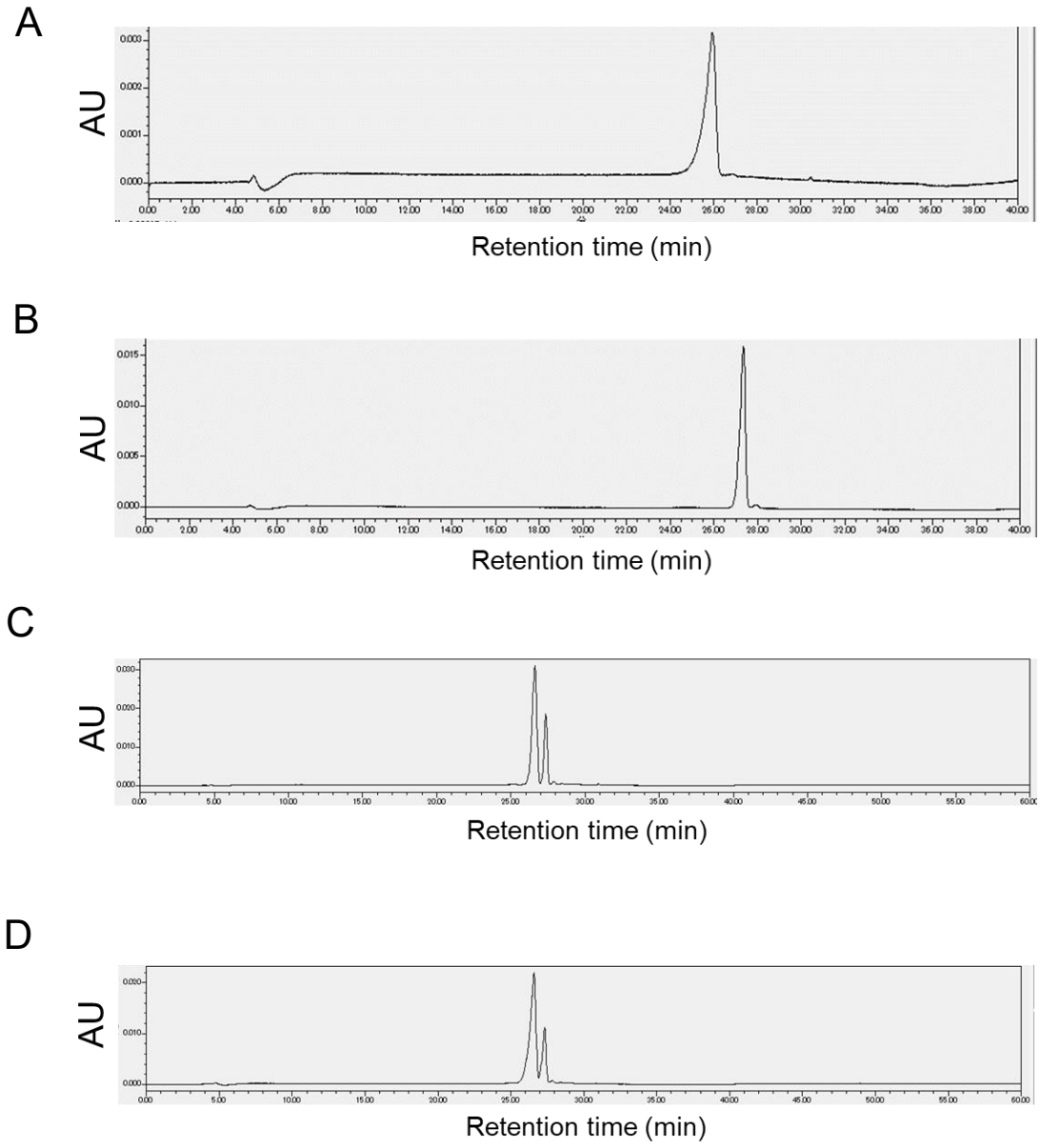


Fig. 4-6-1. Elution pattern of reversed-phase HPLC. (A) C3G. (B) C3R. (C, D) Mulberry fruit extract No. 1 (C) and No. 2 (D).

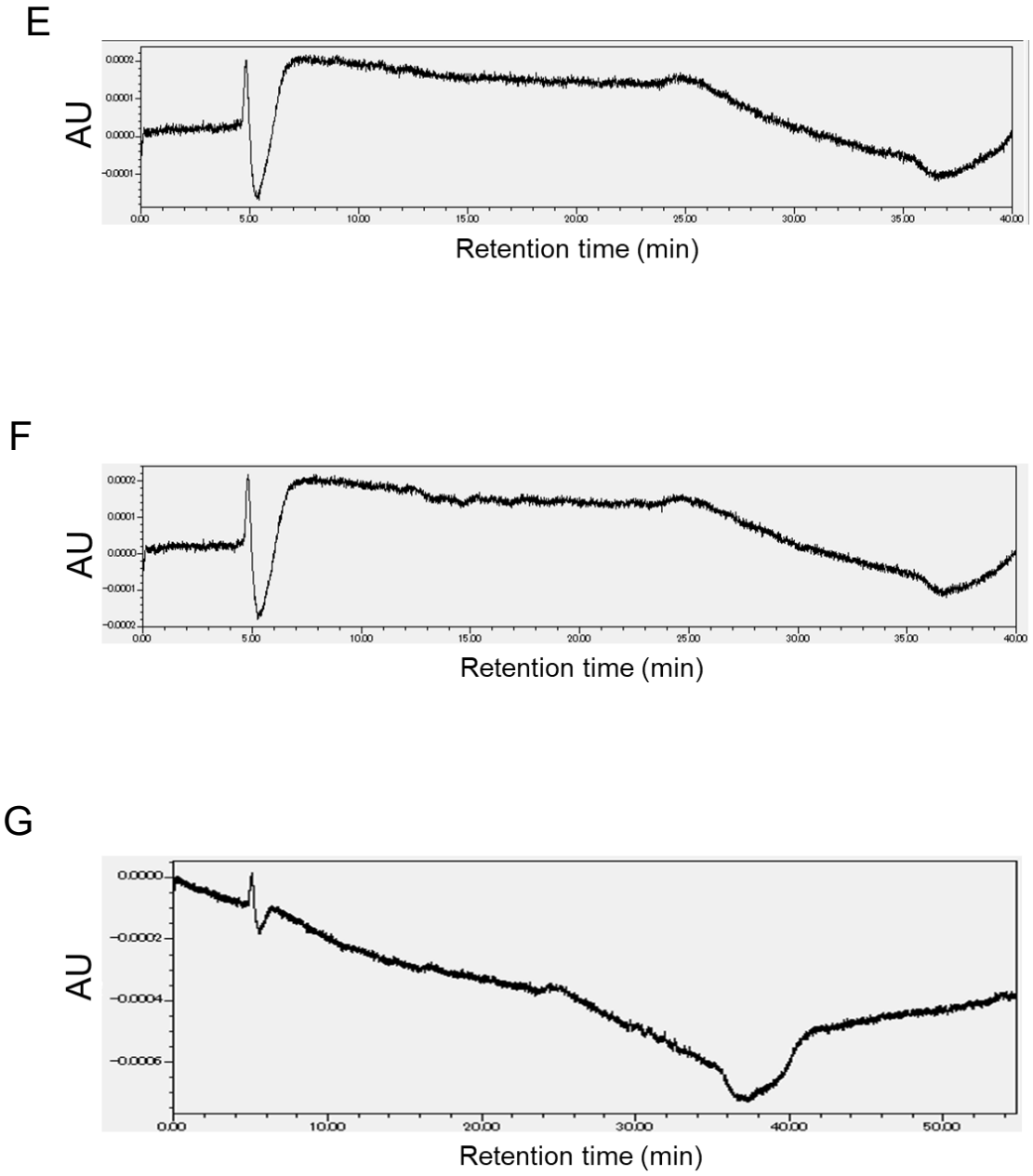


Fig. 4-6-2. Elution pattern of reversed-phase HPLC. (E, F) Mulberry fruit extract No. 3 (E) and No. 4 (F). (G) Powdered leaf extract.

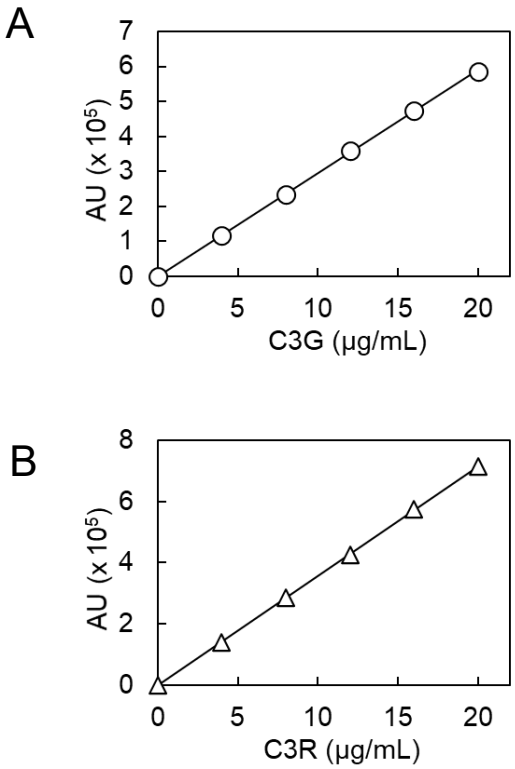


Fig. 4-7. Calibration curves of C3G and C3R. (A) C3G (0–20 μg/mL). (B) C3R (0–20 μg/mL).

Chapter 5

Inhibition of α-amylase and α-glucosidase by *Morus australis* fruit extract and its component iminosugar, anthocyanin, and glucose*

Introduction

Of various parts of the mulberry tree, the leaf and fruit offer the health benefits. Consumption of the leaf tea lowered the postprandial increase in blood glucose concentration in human (18), which contributes to glycemic control. This effect is due to inhibition of α-glucosidase by iminosugar, especially 1-deoxynojirimycin (1-DNJ). In mammals, α-glucosidase exists as mucosal brush border-anchored maltase-glucoamylase (53) and sucrase-isomaltase complexes (54). Consumption of the fruit prevented diabetes and diabetic dementia in mice (27). This effect seemed to be due to certain bioactive components that increased the expression level of brain-derived neurotrophic factor (BDNF) in brain (55). 1-DNJ inhibit α-amylase weekly. In contrast, azadirachtins from *Azadirachta indica*, 4-hydroxyisoleucine from *Trigonella foenum-graecum,* and cinnamaldehyde from *Cinnamomum sieboldii* have been reported to have α-amylase and

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α-glucosidase inhibition activities (56). *Azadirachta indica*, *Trigonella foenum-graecum,* and *Cinnamomum sieboldii* are used in medicinal plants for the management of diabetes although the clinical effectiveness remains controversial (60).

In this study, we characterized the inhibitory effects of *M. australis* fruit extract and its components, iminosugar, anthocyanin, and glucose, against α-amylase and α-glucosidase. We discuss which components in *M. australis* fruit extract are involved in the inhibition of α-glucosidase.

Materials and Methods

Materials – 1-DNJ, fagomine, GAL-DNJ, and *M. australis* leaves were obtained as described in Chapter 2.

Preparation of enzyme solution – α-Amylase from human saliva solution and rat intestinal acetone powder solution were prepared as described in Chapter 3.

Measurement of α-amylase activity –Briefly, 20 μL of 20% w/v soluble starch (Nacalai Tesque), 50 μL 100 mM phosphate buffer (pH 6.0), and 30 μL of *M. australis* fruit extract, C3G, C3R, or glucose solution were mixed and incubated at 37ºC for 10 min. Then, 10 μL of 1.0 mg/mL (70 μg protein/mL) α-amylase solution was added. Blank solution was prepared by adding 10 μL of 0.1 M phosphate buffer (pH 6.0) instead of α-amylase solution. The concentration of reducing sugars of the reaction solution at 1, 2, or 3 min was determined by DNS method using an EnSight multimodal plate reader (PerkinElmer). To estimate the initial reaction rate, maltose was used to make a standard curve.

Measurement of maltase, sucrase, and isomaltase activities –Briefly, 70 μL of 0.1

M phosphate buffer (pH 6.0), 10 μL of maltose, sucrose, or isomaltose solution, and 10 μL of *M. australis* fruit extract, C3G, C3R, or glucose solution were mixed and incubated at 37ºC for 10 min. Then, 10 μL of α-glucosidase solution 35 mg/mL (1.0 mg protein/mL) for measurement of maltase activity or 70 mg/mL (2.1 mg protein/mL) for measurement of sucrase and isomaltase activities and continued at 37ºC. Blank solution was prepared by adding 10 μL of 0.1 M phosphate buffer (pH 6.0) instead of α-glucosidase solution. Glucose concentration of the reaction solution at 1, 2, or 3 min was determined using Glucose CII test (Fujifilm Wako Pure Chemical).

Measurement of glucoamylase activity – Measurement was carried out using as glucoamylase and α-glucosidase assay kit (Kikkoman Corporation) and an EnSight as described previously in Chapter 3. Briefly, the initial reaction rate for glucoamylase (starch hydrolase releasing β-glucose from the non-reducing ends of starch) activity (A) and other α-glucosidase (aryl-glucosides hydrolase releasing β-glucose) activity (B) were determined, separately. The initial reaction rate for A was calculated by reducing B from A+B.

Statistical analysis – Experiments were conducted two times on different days. Values represent the average of triplicate determination. Error bars in figures indicate SD values.

Results and Discussion

Inhibition of α-amylase and α-glucosidase by M. australis fruit extract – We first examined the inhibitory effects of four *M. australis* fruit extracts (No. 1–4) against human α-amylase and maltase-, glucoamylase-, sucrase-, and isomaltase-mediated activities of

rat intestinal acetone powder. Figure 5-1A shows the initial reaction rates of 100 μg/mL α-amylase in the hydrolysis of soluble starch in the presence of varying concentrations of No. 1–4 at pH 6.0 at 37ºC. The initial reaction rates decreased with increasing the concentration of each extract. The IC_{50} values were 10 mg/mL for No. 2 and >15 mg/mL for the other three (Table 1), which was in contrast to our previous result that *M. australis* leaf extract did not inhibit α-amylase.

We next examined the inhibitory effects of No. 1–4 against α-glucosidase. Figure 5-1B–E show the initial reaction rates in the hydrolysis of maltose, G2-β-PNP, sucrose, and isomaltose, respectively, in the presence of varying concentrations of No. 1–4 at pH 6.0 at 37ºC. The initial reaction rates decreased with increasing the concentration of each extract. The IC_{50} values were 1.1–1.7 mg/mL for maltase, 6.9–8.6 mg/mL for glucoamylase, 0.13–1.0 mg/mL for sucrase, and 0.46–1.4 mg/mL for isomaltase (Table 1). When the IC_{50} values of *M. australis* powdered leaf extract (Table 1) were set as 100%, the IC_{50} values of fruit extracts were in the range of 310–470% for maltase, 150–190% for glucoamylase, 260–2,000% for sucrase, and 190–580% for isomaltase (Table 1), indicating that the inhibitory effect of *M. australis* fruit extract was weaker than that of powdered leaf extract. As described above, the IC_{50} values of *M. australis* fruit extract (C) were >10 mg/mL for α-amylase, 1.1–1.7 mg/mL for maltase, 6.9–8.6 mg/mL for glucoamylase, 0.13–1.0 mg/mL for sucrase, and 0.46–1.4 mg/mL for isomaltase (Table 1), whereas the 1-DNJ concentrations in the *M. australis* fruit (D) were 0.04–0.29 mg/g (Chapter 3). Thus, the 1-DNJ concentrations in the *M. australis* fruit extracts that exhibited 50% inhibition (C×D) were calculated to be $>6.0\times10^{-4}$ mg/mL for α-amylase, 6.8×10^{-5}–3.2×10^{-4} mg/mL for maltase, 3.4×10^{-4}–20×10^{-4} mg/mL for glucoamylase, 3.7×10^{-5}–4.0×10^{-5} mg/mL for sucrase, and 5.6×10^{-5}–1.3×10^{-4} mg/mL for isomaltase

(Table 2). Compared with the IC_{50} values of 1-DNJ, these values were >0.0050% for α-amylase, 9.7–46% for maltase, 3.1–18% for glucoamylase, 27–29% for sucrase, and 8.6–21% for isomaltase (Table 2), indicating that these values were comparable to IC_{50} values for maltase, glucoamylase, sucrase, and isomaltase, but not for α-amylase.

In a similar way, the fagomine and GAL-DNJ concentrations in the fruit extracts that exhibited 50% inhibition were calculated (Table 3 and 4, respectively). Compared with the IC_{50} values, these values were >0.027% for α-amylase, 0.083–0.23% for maltase, 0.014–0.049% for glucoamylase, 0.063–0.37% for sucrase, and 0.71–1.9% for isomaltase in fagomine (Table 3) and 0.025–0.030% for maltase, 0.025–0.032% for glucoamylase, 0.025–0.12% for sucrase, and 0.034–0.075% for isomaltase in GAL-DNJ (Table 4), indicating that unlike the case with 1-DNJ, they were not comparable to the IC_{50} values. These results suggested that in the *M. australis* fruit extract, 1-DNJ is not a main inhibitor of α-amylase, but is a main inhibitor of α-glucosidase, and that neither fagomine nor GAL-DNJ is a main inhibitor of α-amylase or α-glucosidase.

Amézqueta et al. reported that orally-administered fagomine in rats was partly absorbed but mostly excreted in feces within 24 h (25). Takasu et al. reported that orally-administered GAL-DNJ in rats is partly degraded into galactose and 1-DNJ (50). Considering these results, the contribution of fagomine and GAL-DNJ to the inhibition of α-glucosidase cannot be underestimated from our results.

Besides *Morus* fruit, sweet cheery contains various bioactive compounds and has great attention as a healthy sweetener (61). Gonçalves et al. identified 23 phenolic compounds in five varieties of sweet cherries (59). In that study, they showed that the IC_{50} value of the most active sweet cheery extract for α-glucosidase was 0.013 mg/mL, which corresponded to 0.42 mg/mL when the average yield after drying (3.1%) was

considered (60) and was comparable to the IC_{50} values of *M. australis* fruit extract (0.13–8.6 mg/mL) obtained in this study (Table 1).

Inhibition of α-amylase and α-glucosidase by anthocyanin and quantification of anthocyanin in M. australis fruit – Anthocyanin is a subgroup of flavonoid. Consumption of foods rich in anthocyanins has positive effects on health including a reduced risk of cardiovascular disease and cancer (62–64). Structurally, anthocyanin is the glycosylated form of anthocyanidin. C3G and C3R are the major components of anthocyanin. To explore the contribution of anthocyanin in *M. australis* to the inhibition of α-amylase and α-glucosidase, we examined the inhibitory effects of C3G and C3R towards α-amylase and α-glucosidase. We also quantified the concentrations of C3G and C3R in *M. australis* leaf and fruit.

Figure 5-2A shows the initial reaction rates of 100 μg/mL α-amylase in the hydrolysis of soluble starch in the presence of varying concentrations of C3G or C3R at pH 6.0 at 37ºC. The initial reaction rates were stable with increasing the concentrations of C3G or C3R. Figure 5-2B–E show the initial reaction rates in the hydrolysis of maltose, G2-β-PNP, sucrose, and isomaltose, respectively, in the presence of varying concentrations of C3G or C3R at pH 6.0 at 37ºC. The initial reaction rates decreased with increasing the concentration of C3G or C3R and for maltase (Fig. 5-2B), glucoamylase (Fig. 5-2C), sucrase (Fig. 5-2D), and isomaltase (Fig. 5-2E). The initial reaction rate for glucoamylase did not reach 50% of the initial reaction rate (Fig. 5-2C). These results indicated that C3G and C3R inhibited maltase, sucrase, and isomaltase, partially inhibited glucoamylase, and did not inhibit α-amylase. The IC_{50} values are shown in Table 1.

The C3G and C3R concentrations in the fruit extracts that exhibited 50% inhibition were calculated (Tables 5 and 6, respectively). Compared with the IC_{50} values, these

values were 2.3–6.3% for maltase, 0.41–1.9% for sucrase, and 1.4–4.7% for isomaltase in C3G (Table 5) and 0.60–2.0% for maltase, 0.049–0.27% for sucrase, and 0.37–1.4% for isomaltase in C3R (Table 6). These results suggested that in the *M. australis* fruit extract, anthocyanin contributes to the inhibition of maltase, sucrase, and isomaltase although it does not seem to be a main inhibitor.

Inhibition of α-amylase and α-glucosidase by glucose and quantification of glucose in M. australis fruit – Glucose is abundantly present in fruit. To explore the contribution of glucose in *M. australis* to the inhibition of α-amylase and α-glucosidase, we examined the inhibitory effects of glucose toward α-amylase and α-glucosidase and quantified the concentrations of glucose in *M. australis* leaf and fruit. Figure 5-3A shows the initial reaction rates of 100 μg/mL *α*-amylase in the hydrolysis of soluble starch in the presence of varying concentrations of glucose at pH 6.0 at 37ºC. The initial reaction rates decreased with increasing concentrations of glucose. Figure 5-3B–E show the initial reaction rates in the hydrolysis of maltose, G2-β-PNP, sucrose, and isomaltose, respectively, in the presence of varying concentrations of glucose at pH 6.0 at 37ºC. The initial reaction rates decreased with increasing concentration of glucose. They reached almost zero for maltase (Fig. 5-3B), sucrase (Fig. 5-3D), and isomaltase (Fig. 5-3E), but not for glucoamylase (Fig. 5-3C). The IC_{50} values for α-amylase, maltase, sucrase, and isomaltase are shown in Table 1. Quantification of glucose in the fruit extract using Glucose CII test (Fujifilm Wako Pure Chemical) revealed that the glucose concentrations were 262 mg/g for the dried fruit No. 1, 123 mg/g dried fruit for No. 2, 260 mg/g dried fruit for No. 3, 211 mg/g dried fruit for No. 4, and 0 mg/g for the powdered leaf (Chapter 4).

The glucose concentrations in the fruit that exhibited 50% inhibition were calculated (Table 7). When the each IC_{50} value of glucose was set as 100%, these values were 21–

68% for α-amylase, 20–64% for maltase, 1.5–24% for sucrase, and 10–66% for isomaltase (Table 7). These results indicated that in the *M. australis* fruit extract, glucose is a main inhibitor of α-amylase, maltase, sucrase, and isomaltase. Therefore, unlike *M. australis* leaf, these inhibitory effects of *M. australis* fruit do not mean that it is suitable to lower the postprandial increase in blood glucose level.

Conclusion – Besides iminosugar, anthocyanin, and glucose, various compounds such as flavonoids, phenolic acids, saponins, carotenoids have been reported to inhibit α-amylase and α-glucosidase (64). However, our results indicated that in *M. australis* fruit, 1-DNJ, anthocyanin, and glucose are involved in the inhibition of α-amylase and α-glucosidase. This is in contrast to that in *M. australis* leaf, 1-DNJ is a main inhibitor of α-glucosidase, and neither anthocyanin nor glucose are contained. Considering that the *M. australis* fruit contains high amounts of glucose, unlike *M. australis* leaf, it cannot be ingested to suppress postprandial hyperglycemia. However, *M. australis* fruit is better than various sweeteners including sucrose and glucose, because it contains iminosugars and anthocyanin. Our results encourage the application of *M. australis* fruit as a healthy sweetener.

Table 1. IC_{50} values of the fruit and powdered leaf extracts, iminosugar, anthocyanin, and glucose.

	α-Amylase		Maltase		Glucoamylase		Sucrase		Isomaltase	
	(mM)	(mg/mL)	(mM)	(mg/mL)	(mM)	(mg/mL)	(mM)	(mg/mL)	(mM)	(mg/mL)
Fruit extract #1	—	>15	—	1.1 (310%)[a]	—	7.8 (170%)	—	0.22 (440%)	—	0.55 (230%)
Fruit extract #2	—	10	—	1.1 (310%)	—	6.9 (150%)	—	0.13 (260%)	—	0.46 (190%)
Fruit extract #3	—	>15	—	1.7 (470%)	—	8.6 (190%)	—	1.0 (2,000%)	—	1.4 (580%)
Fruit extract #4	—	>15	—	1.5 (420%)	—	8.2 (180%)	—	0.22 (440%)	—	0.60 (250%)
Powdered leaf extract	—	No inhibition	—	0.36 (100%)	—	4.5 (100%)	—	0.050 (100%)	—	0.24 (100%)
1-DNJ	78	13	4.3×10^{-3}	7.0×10^{-4}	0.066	0.011	8.6×10^{-4}	1.4×10^{-4}	4.0×10^{-3}	6.5×10^{-4}
Fagomine	38	5.6	1.2	0.18	41	5.9	0.24	0.035	0.075	0.011
GAL-DNJ	No inhibition	No inhibition	8.9	2.9	53	17	1.3	0.41	4.8	0.91
C3G	No inhibition	No inhibition	0.43	0.21	No inhibition	No inhibition	0.29	0.14	0.29	0.14
C3R	No inhibition	No inhibition	0.35	0.22	No inhibition	No inhibition	0.51	0.32	0.24	0.15
Glucose	32	5.8	3.8	0.69	No inhibition	No inhibition	6.1	1.1	3.1	0.55

Values for the inhibition of α-amylase and α-glucosidase by 1-DNJ, fagomine, GAL-DNJ, and powdered leaf extract are from Chapter 2.

[a]Values in parenthesis indicate relative values (%) compared to powdered leaf extract.

Table 2. Calculated 1-DNJ concentration in the fruit and powdered leaf extracts that exhibit 50% inhibition.

	α-Amylase (mg/mL)	Maltase (mg/mL)	Glucoamylase (mg/mL)	Sucrase (mg/mL)	Isomaltase (mg/mL)
Fruit extract #1	$>2.6\times10^{-3}$ (0.020%)[a]	1.9×10^{-4} (27%)	1.3×10^{-3} (12%)	3.7×10^{-5} (27%)	9.4×10^{-5} (14%)
Fruit extract #2	2.9×10^{-3} (0.022%)	3.2×10^{-4} (46%)	2.0×10^{-3} (18%)	3.8×10^{-5} (27%)	1.3×10^{-4} (21%)
Fruit extract #3	$>6.0\times10^{-4}$ (0.0046%)	6.8×10^{-5} (9.7%)	3.4×10^{-4} (3.1%)	4.0×10^{-5} (29%)	5.6×10^{-5} (8.6%)
Fruit extract #4	$>2.7\times10^{-3}$ (0.021%)	2.7×10^{-4} (39%)	1.5×10^{-3} (13%)	4.0×10^{-5} (28%)	1.1×10^{-4} (17%)
Powdered leaf extract	No inhibition	1.4×10^{-3} (206%)	1.8×10^{-2} (164%)	2.0×10^{-4} (143%)	9.6×10^{-4} (148%)

[a]Values in parenthesis indicate relative values (%) compared to the IC_{50} values of 1-DNJ (Table 1).

Table 3. Calculated fagomine concentration in the fruit and powdered leaf extracts that exhibit 50% inhibition.

	α-Amylase (mg/mL)	Maltase (mg/mL)	Glucoamylase (mg/mL)	Sucrase (mg/mL)	Isomaltase (mg/mL)
Fruit extract #1	$>5.6\times10^{-3}$ (0.099%)[a]	4.1×10^{-4} (0.23%)	2.9×10^{-3} (0.049%)	8.1×10^{-5} (0.23%)	2.0×10^{-4} (1.9%)
Fruit extract #2	1.7×10^{-3} (0.030%)	1.9×10^{-4} (0.10%)	1.2×10^{-3} (0.020%)	2.2×10^{-5} (0.063%)	7.8×10^{-5} (0.71%)
Fruit extract #3	$>2.0\times10^{-3}$ (0.035)	2.2×10^{-4} (0.12%)	1.1×10^{-3} (0.019%)	1.3×10^{-4} (0.37%)	1.8×10^{-4} (1.7%)
Fruit extract #4	$>1.5\times10^{-3}$ (0.027%)	1.5×10^{-4} (0.083%)	8.2×10^{-4} (0.014%)	2.2×10^{-5} (0.063%)	6.0×10^{-5} (0.55%)
Powdered leaf extract	No inhibition	1.7×10^{-4} (0.092%)	2.1×10^{-3} (0.035%)	2.3×10^{-5} (0.066%)	1.1×10^{-4} (1.0%)

[a]Values in parenthesis indicate relative values (%) compared to the IC_{50} values of fagomine (Table 1).

Table 4. Calculated GAL-DNJ concentration in the fruit and powdered leaf extracts that exhibit 50% inhibition.

	α-Amylase (mg/mL)	Maltase (mg/mL)	Glucoamylase (mg/mL)	Sucrase (mg/mL)	Isomaltase (mg/mL)
Fruit extract #1	$>9.8\times10^{-3}$	7.1×10^{-4} (0.025%)[a]	5.1×10^{-3} (0.030%)	1.4×10^{-4} (0.035%)	3.6×10^{-4} (0.039%)
Fruit extract #2	7.8×10^{-3}	8.6×10^{-4} (0.030%)	5.4×10^{-3} (0.032%)	1.0×10^{-4} (0.025%)	3.6×10^{-4} (0.039%)
Fruit extract #3	$>7.4\times10^{-3}$	8.3×10^{-4} (0.029%)	4.2×10^{-3} (0.025%)	4.9×10^{-4} (0.12%)	6.9×10^{-4} (0.075%)
Fruit extract #4	$>7.8\times10^{-3}$	7.8×10^{-4} (0.027%)	4.3×10^{-3} (0.025%)	1.1×10^{-4} (0.028%)	3.1×10^{-4} (0.034%)
Powdered leaf extract	No inhibition	9.0×10^{-4} (0.031%)	0.011 (0.066%)	1.3×10^{-4} (0.030%)	6.0×10^{-4} (0.066%)

[a]Values in parenthesis indicate relative values (%) compared to the IC_{50} values of GAL-DNJ (Table 1).

Table 5. Calculated C3G concentrations in the fruit and powdered leaf extracts that exhibit 50% inhibition.

	α-Amylase (mg/mL)	Maltase (mg/mL)	Glucoamylase (mg/mL)	Sucrase (mg/mL)	Isomaltase (mg/mL)
Fruit extract #1	0.18	0.013 (6.3%)[a]	0.094	2.6×10^{-3} (1.9%)	6.6×10^{-3} (4.7%)
Fruit extract #2	0.044	4.8×10^{-3} (2.3%)	0.030	5.7×10^{-4} (0.41%)	2.0×10^{-3} (1.4%)
Fruit extract #3	0	0	0	0	0
Fruit extract #4	0	0	0	0	0
Powdered leaf extract	0	0	0	0	0

[a]Values in parenthesis indicate relative values (%) compared to the IC_{50} values of C3G (Table 1).

Table 6. Calculated C3R concentrations in the fruit and powdered leaf extracts that exhibit 50% inhibition.

	α-Amylase (mg/mL)	Maltase (mg/mL)	Glucoamylase (mg/mL)	Sucrase (mg/mL)	Isomaltase (mg/mL)
Fruit extract #1	0.059	4.3×10^{-3} (2.0%)[a]	0.030	8.6×10^{-4} (0.27%)	2.1×10^{-3} (1.4%)
Fruit extract #2	0.012	1.3×10^{-3} (0.60%)	8.3×10^{-3}	1.6×10^{-4} (0.049%)	5.5×10^{-4} (0.37%)
Fruit extract #3	0	0	0	0	0
Fruit extract #4	0	0	0	0	0
Powdered leaf extract	0	0	0	0	0

[a]Values in parenthesis indicate relative values (%) compared to the IC_{50} values of C3R (Table 1).

Table 7. Calculated glucose concentrations in the fruit and powdered leaf extracts that exhibit 50% inhibition.

	α-Amylase (mg/mL)	Maltase (mg/mL)	Glucoamylase (mg/mL)	Sucrase (mg/mL)	Isomaltase (mg/mL)
Fruit extract #1	3.9 (68%)[a]	0.29(42%)	2.0	0.058(5.2%)	0.14(26%)
Fruit extract #2	1.2 (21%)	0.14(20%)	0.85	0.016(1.5%)	0.057(10%)
Fruit extract #3	3.9 (67%)	0.44(64%)	2.2	0.26(24%)	0.36(66%)
Fruit extract #4	3.2 (55%)	0.32(46%)	1.7	0.046(4.2%)	0.13(23%)
Powdered leaf extract	0	0	0	0	0

[a]Values in parenthesis indicate relative values (%) compared to the IC_{50} values of glucose (Table 1).

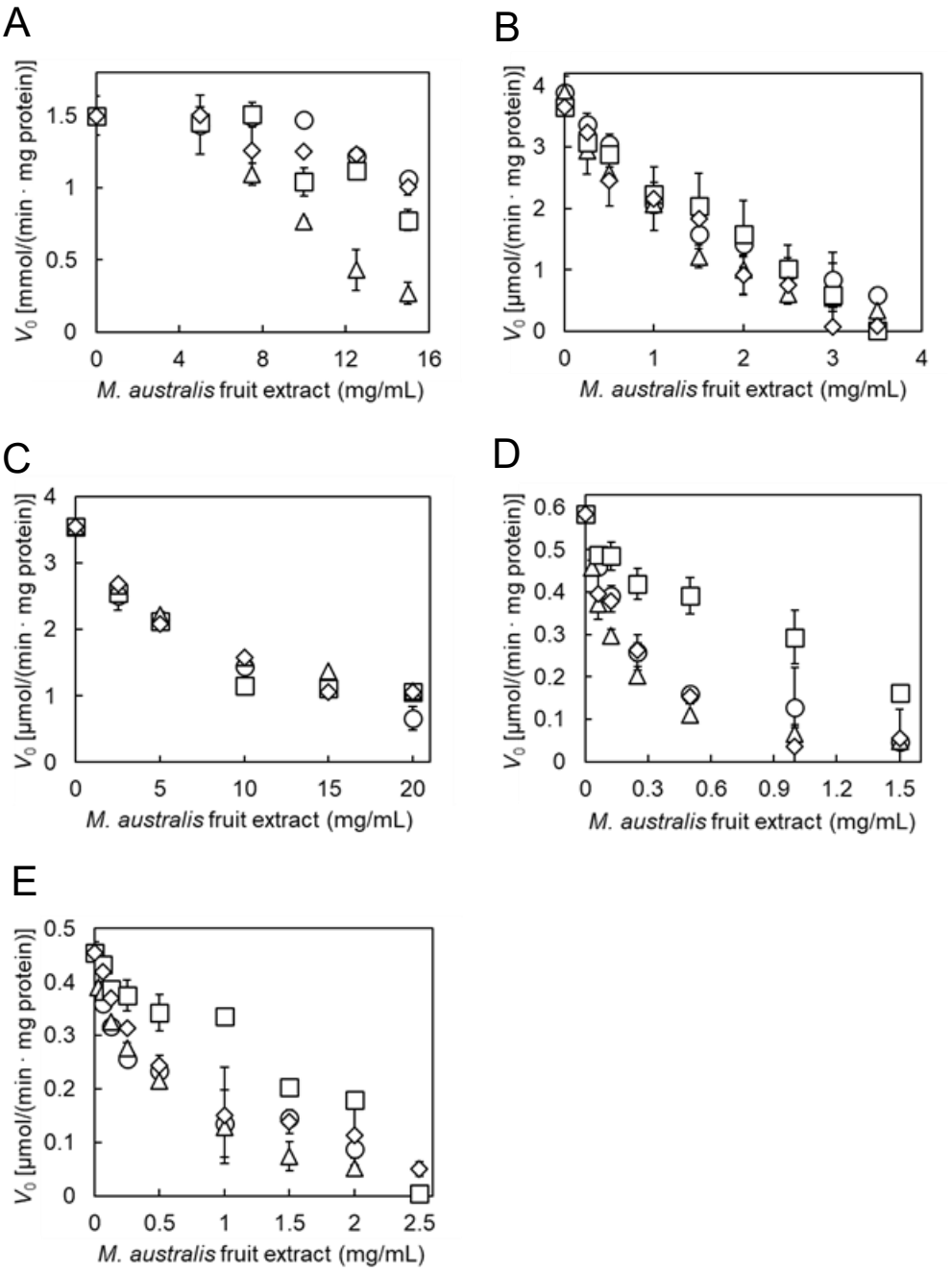

Fig. 5-1. Effects of fruit extract concentration on the hydrolysis by human α-amylase or rat intestinal acetone powder. The reaction was carried out at pH 6.0 at 37ºC with 0.7 µg protein/mL human α-amylase and 36 mg/mL soluble starch (A), with 0.10 mg protein/mL rat intestinal acetone powder solution and 50 mM maltose (B) or 15 mM G2-β-PNP (C), or with 0.21 mg protein/mL rat intestinal acetone powder solution and 100 mM sucrose (D) or 50 mM isomaltose (E). Extract of *M. australis* fruit No. 1 (open circle), 2 (open triangle), 3 (open square), and 4 (open diamond). Error bars indicate SD values. The average of triplicate determination is shown.

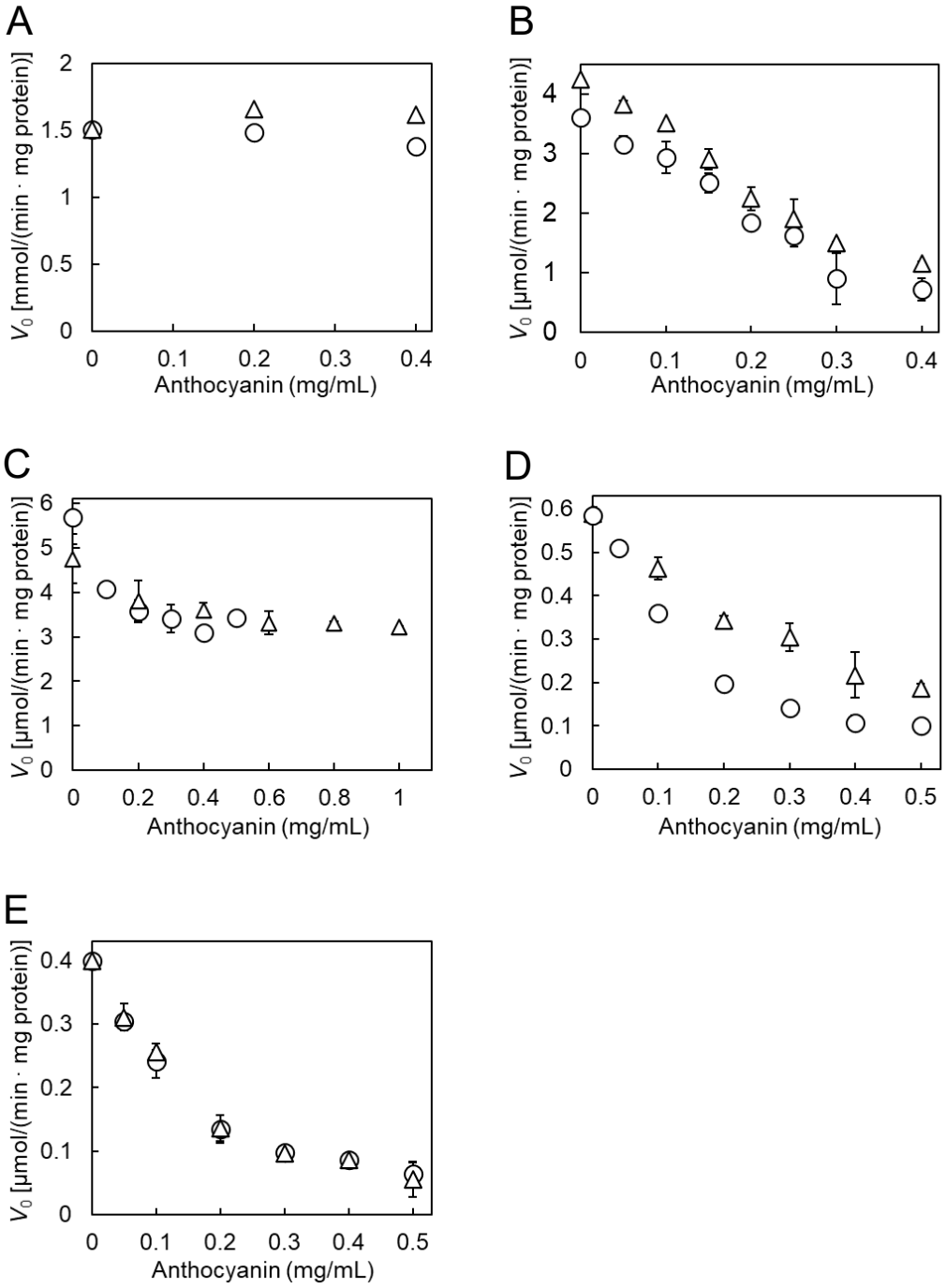

Fig. 5-2. Effects of C3G and C3G concentrations on the hydrolysis by human α-amylase or rat intestinal acetone powder. (A) The reaction was carried out at pH 6.0 at 37ºC with 0.7 μg protein/mL human α-amylase and 36 mg/mL soluble starch (A), with 0.10 mg protein/mL rat intestinal acetone powder solution and 50 mM maltose (B) or 15 mM G2-β-PNP (C), or with 0.21 mg protein/mL rat intestinal acetone powder solution and 100 mM sucrose (D) or 50 mM isomaltose (E). Symbols: C3G (open circle) and C3R (open triangle). Error bars indicate SD values. The average of triplicate determination is shown.

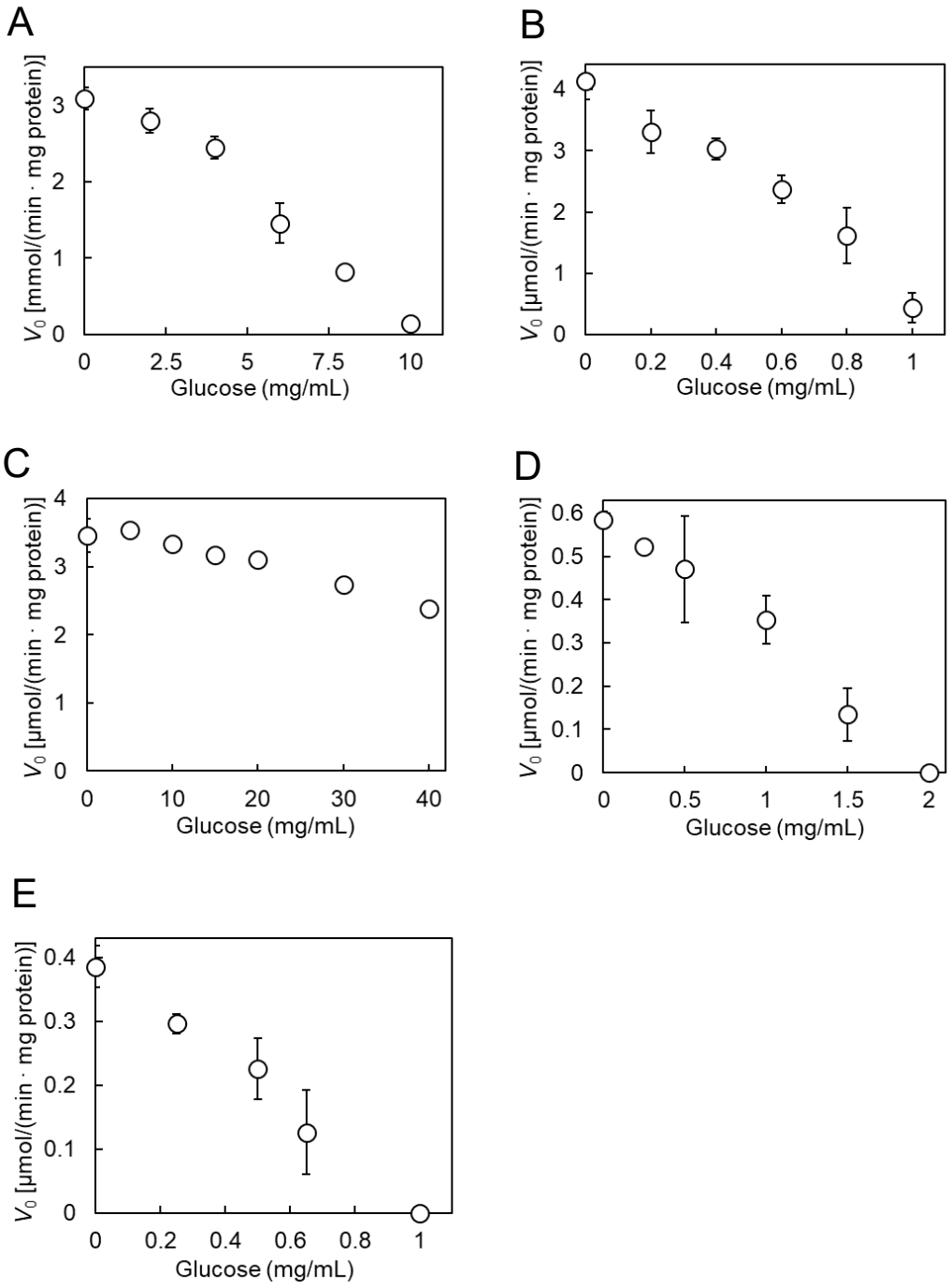

Fig. 5-3. Effects of the glucose concentrations on the hydrolysis by human α-amylase or rat intestinal acetone powder. (A) The reaction was carried out at pH 6.0 at 37ºC with 0.7 µg protein/mL human α-amylase and 36 mg/mL soluble starch (A), with 0.10 mg protein/mL rat intestinal acetone powder solution and 50 mM maltose (B) or 15 mM G2-β-PNP (C) or with 0.21 mg protein/mL rat intestinal acetone powder solution and 100 mM sucrose (D) or 50 mM isomaltose (E). Error bars indicate SD values. The average of triplicate determination is shown.

Summary

Chapter 1

We quantified iminosugars in *Morus australis* leaves. By LC-MS/MS, the concentrations of 1-DNJ, fagomine, and GAL-DNJ in the powdered leaves were 4.0, 0.46, and 2.5 mg/g, respectively, and those in the roasted ones were 1.0, 0.24, and 0.73 mg/g, respectively. The iminosugar concentrations in the roasted leaves were 25–52% of those in the powdered leaves, suggesting that the roasting process considerably degraded iminosugars. 1-DNJ concentration in the leaves was 1.6–8.7 and 1.4–4.2 fold higher than the fagomine and GAL-DNJ concentrations, respectively.

Chapter 2

We performed the kinetic analysis of the inhibitory effects of powdered or roasted *Morus australis* leaf extract and its component iminosugars, such as 1-DNJ, fagomine, and GAL-DNJ on the activities of α-amylase and α-glucosidase. Each leaf extract sample inhibited all α-glucosidase-related enzymes (maltase, glucoamylase, sucrase, and isomaltose), but not α-amylase. 1-DNJ and fagomine showed weak α-amylase inhibitory activity while GAL-DNJ exhibited none. 1-DNJ showed a strong α-glucosidase inhibitory potential. The inhibitory potential against these α-glucosidases was 18–500 and 1,500–3,000-fold higher in the case of 1-DNJ than that observed in the case of fagomine and GAL-DNJ, respectively. At pH 6.0 at 37ºC, the IC_{50} values of the extracts from the boiled powdered or roasted leaves were 0.36 and 1.1 mg/mL, respectively. At the same condition, the IC_{50} values of 1-DNJ, fagomine, and GAL-DNJ were 0.70 μg/mL, 0.18 mg/mL, and 2.9 mg/mL, respectively. These results suggested that in *M. australis*, 1-DNJ is a major

inhibitor of α-glucosidase. The K_i values of the extract of *M. australis* powdered leaves and 1-DNJ are temperature- and pH-dependent. *M. australis* leaves contain 1-DNJ a lot and strongly inhibit α-glucosidase activity. Steady-state kinetic analysis revealed that the powdered and roasted leaves exhibited competitive inhibition. We also observed that the indigestible dextrin could considerably inhibit α-amylase. When the powdered *M. australis* leaf extract was blended with indigestible dextrin, the mixture inhibited α-amylase, as well as maltase, glucoamylase, sucrase, and isomaltase. These results suggest that the ingestion of the leaf extract blended with indigestible dextrin might have the potential to efficiently suppress the postprandial blood glucose level increase.

Chapter 3

We quantified iminosugar, anthocyanin, and glucose in *M. australis* fruit (No 1, 2, 3, and 4) extracts. Based on the calibration curves, the concentrations of iminosugar in the dried *M. australis* fruit were 0.04–0.29 mg/g for 1-DNJ, 0.10–0.37 mg/g for fagomine, and 0.49–0.78 mg/g for GAL-DNJ. When the concentration of 1-DNJ in the powdered *M. australis* leaf was set 100%, those in *M. australis* fruit were 1.0–7.3%. *M. australis* fruit No. 1 and 2 exhibited the peak corresponding to cyanidin-3-glucoside (C3G) and cyanidin-3-rutinoside (C3R) while No. 3 or 4 or the powdered leaf extract did not. Based on the calibration curves, the concentrations of C3G and C3R were 12 and 3.9 mg/g dried fruit, respectively, for No. 1 and 4.4 and 1.2 mg/g dried fruit, respectively, for No. 2. These results suggested that the concentration of anthocyanin in *M. australis* fruit depends on strains considerably.

Chapter 4

We examined the inhibitory effects of *Morus australis* fruit extract and its components, that is, three iminosugars (1-DNJ, fagomine, and GAL-DNJ), two anthocyanins (C3G and C3R), and glucose, against α-amylase and α-glucosidase. The IC_{50} values of the fruit extracts of our *M. australis* subspecies were >10 mg/mL for α-amylase, 1.1–1.7 mg/mL for maltase, 6.9–8.6 mg/mL for glucoamylase, 0.13–1.0 mg/mL for sucrase, and 0.46–1.4 mg/mL for isomaltase. When the IC_{50} value of each component and the concentration of each component in the fruit extract were taken into consideration, our results indicated that glucose are involved in the inhibition of α-amylase, and 1-DNJ and glucose are involved in the inhibition of α-glucosidase. This is in contrast to that in *M. australis* leaf, neither anthocyanin nor glucose are contained, and 1-DNJ is a main inhibitor.

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List of publications

Original Papers

1. Qiao, Y., Nakayama, J., Iketuchi, T., Ito, M., Kimura, T., Kojima, K., Takita, T., and Yasukawa, K. (2020) Kinetic analysis of inhibition of α-glucosidase by leaf powder from *Morus australis* and its component iminosugars. *Biosci. Biotechnol. Biochem.,* **84**, 2149–2156
2. Qiao, Y., Ito, M., Kimura, T., Ikeuchi, T., Takita, T., and Yasukawa, K. (2021) Inhibitory effect of *Morus australis* leaf extract and its component iminosugars on intestinal carbohydrate-digesting enzymes. *J. Biosci. Bioeng*., **132**, 226–233
3. Qiao, Y., Ikeda, Y., Ito, M., Kimura, T., Ikeuchi, T., Takita, T., and Yasukawa, K. (2022) Inhibition of α-amylase and α-glucosidase by *Morus australis* fruit extract and its components iminosugar, anthocyanin, and glucose. *J. Food Sci*., **87**, 1672–1683

Related Papers

1. Kume, D., Qiao, Y., Nakayama, J., Shimajiri, Y., and Ito, M. (2021) Inhibitory effect of bread containing powdered *Morus australis* leaves on postprandial elevation of blood glucose, *J. Nutr. Sci. Vitaminol*., **74**, 15–20
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