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Article

Inhibition of α-glucosidase activity by *Morus australis* fruit food

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Mulberry fruit is thought as a healthy sweetener because it contains iminosugars, such as 1-deoxynojirimycin (1-DNJ), fagomine and 2-*O*-α-D-galactopyranosyl deoxynojirimycin (GAL-DNJ), that inhibits α-glucosidase. In this study, we quantified iminosugars and glucose concentrations in *Morus australis* fruit foods and analyzed their inhibitory effects against the activities of α-glucosidase-related four enzymes, maltase, glucoamylase, sucrase, and isomaltase. By LC-MS/MS, the concentrations of 1-DNJ, fagomine, and GAL-DNJ of the *M. australis* fruit wine, juice, vinegar drink, vinegar, and jam were 1.76–32.5, 0.058–0.290, and 30.2–209 μg/mL, respectively. By high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD), the glucose concentrations were 0 mg/mL for wine and 13.9–39.7 mg/mL for juice, vinegar drink, vinegar, and jam. The IC_{50} values of these five foods were 1.9–4.4 % v/v for maltase, 0.37-5.0 % for glucoamylase, and 0.28–1.2 % for sucrase, and 2.0% for isomaltase. Considering the 1-DNJ and glucose concentrations in these five *M. australis* fruit foods and the IC_{50} values of 1-DNJ and glucose for α-glucosidase, it was suggested that 1-DNJ is a main inhibitor for α-glucosidase in these foods, and glucose is involved in the inhibition except for wine.

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Key words: α-glucosidase; 1-DNJ; glucose; *Morus australis*; fruit.

Introduction

α-Glucosidase [EC 3.2.1.20], along with α-amylase, is involved in the digestion of sugars. It catalyzes the hydrolysis reaction of α-1,4 glycosidic bonds at the non-reducing end of the substrate. In mammals, α-glucosidase exists as mucosal brush border-anchored maltase-glucoamylase [1] and sucrase-isomaltase complexes [2]. It is widely accepted that inhibition of α-glucosidase is one of the strategies

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to treat type-2 diabetes. Inhibitory substances toward α-glucosidase are attracting attention. Among them, 1-deoxynojirimycin (1-DNJ), an iminosugar that exists in mulberry leaf, has a potent inhibitory activity [3–7].

Morus australis is one of mulberry species, distributed in Ryukyu islands and cultivated as a crop for silkworm feed, fruit, and timber. In comparison with *Morus* sp. that is distributed in Japanese main island, *M. australis* contains more 1-DNJ [8]. The intake of *M. australis* leaf tea suppressed sucrose-induced elevation of blood glucose and insulin levels in human [8, 9]. By LC-MS/MS, the concentrations of 1-DNJ and two other iminosugars, fagomine and 2-*O*-α-D-galactopyranosyl deoxynojirimycin (GAL-DNJ), in the *M. australis* powdered leaf extract were comparable (4.0, 0.46, and 2.5 mg/g, respectively) [10]. However, the IC_{50} values of 1-DNJ against maltase, glucoamylase, sucrase, and isomaltase were 1.4×10^{-4}–0.011 mg/mL, being 19–620 and 800–2,100-fold smaller than those of fagomine and GAL-DNJ, respectively, showing that 1-DNJ is a major inhibitor of α-glucosidase [11].

Mulberry fruit is recognized as a healthy sweetener because it contains various compounds such as anthocyanins, flavonoids, phenolic acids, saponins, and carotenoids. Consumption of mulberry fruit prevented diabetes and diabetic dementia in mice [12]. However, little has been known about the inhibition of α-glucosidase by compounds contained in mulberry fruit. We previously reported that the 1-DNJ concentration in the *M. australis* dried fruit were 0.04–0.29 mg/g, which was considerably smaller than that in the *M. australis* leaf (4.0 mg/g) [11,13]. The glucose concentration in the *M. australis* dried fruit was 123–262 mg/g [13]. We also reported that 1-DNJ and glucose are the components in *M. australis* fruit, that are involved in the inhibition of maltase, glucoamylase, sucrase, and isomaltase [13].

Various mulberry fruit foods have been developed. They include wine, juice, vinegar drink, vinegar, and jam. Intake of such food is expected to inhibit α-glucosidase activity and suppress postprandial elevation of blood glucose. The aim of this study is to quantify iminosugars and glucose in *M. australis* fruit foods and characterize their inhibitory effects against α-glucosidase *in vitro*.

Materials and Methods

Materials　Five *M. australis* fruit foods (red wine [Product name: Mūre de soleil] produced in MAOI corporation [Yubari, Hokkaido], juice [Mulberry fruit juice], vinegar drink [Mulberry fruit vinegar drink], and jam [Mulberry fruit jam] each produced in Okinawa Plantation Corporation [Urasoe, Okinawa], and vinegar [Mulberry fruit vinegar] produced in Ryukyu cement Corporation [Nago, Okinawa]) were purchased from Okinawa Biken Sale Corporation (Shimajiri, Okinawa). Three non-mulberry red wines (Cardounettes Cabernet Sauvignon/Merlot produced in Les Vignobles Foncalieu (France), Conosur Cabernet Sauvignon Bicicleta Reserva produced in Vina Cono Sur (Chile), and Biwa Wine produced in Ota Sake Brewery Corporation (Kusatsu, Japan)) were purchased from liquor shop. They are abbreviated as Wine #1, #2, and #3, respectively. Maltose, 1-DNJ, and Glucose CII test were purchased from Fujifilm Wako Pure Chemical (Osaka, Japan). Sucrose was purchased from Nacalai Tesque (Kyoto, Japan). Isomaltose was purchased from Tokyo Kasei (Tokyo, Japan). Fagomine and GAL-DNJ were purified from mulberry leaves as reported previously [14].

Liquid chromatography-MS/MS (LC-MS/MS) analysis　The jam (2.5 g) was mixed with 5 mL of 50% ethanol and then sonicated. The wine, juice, vinegar drink, vinegar and sonicated jam

(each 1.5 mL) were centrifuged at 10,000 × *g* for 5 min at 4ºC. The supernatant was filtered with Syringe Filter PES 0.22 μm/φ32 mm (As One, Osaka, Japan). The filtrates were diluted with 0.1% formic acid, 50% v/v acetonitrile to be 2% v/v for wine, 5% v/v for juice, vinegar, and jam, and 10% for vinegar drink for the quantification of 1-DNJ and to be 0.1% for the quantification of GAL-DNJ. Standard 1-DNJ, fagomine, and GAL-DNJ were dissolved in 0.1% formic acid, 50% acetonitrile to be 200–1,000 ng/mL. 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 for 1-DNJ and 50%–60% B for fagomine and GAL-DNJ; 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 postcolumn 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 *M. australis* fruit food were calculated from calibration curves using standard 1-DNJ, fagomine, and GAL-DNJ.

HPAE-PAD analysis The wine, juice, vinegar drink, vinegar, and sonicated jam (each 100 μL) were diluted with acetonitrile (100 μL) and then centrifuged at 15,000 × *g* at 4 ºC for 5 min. The supernatants were diluted with water to be 0.025% for wine, vinegar and jam, and 0.005% for juice and vinegar drink and filtered with Syringe Filter PES 0.22 μm/φ32 mm. Conditions of HPAE-PAD are as follows: apparatus, Thermo Scientific Dionex Ion Chromatography ICS-5000K (Thermo Fisher Scientific, Waltham, MA); guard column, Dionex CarboPac PA210-Fast-4 guard column (2.0 × 30 mm, Thermo Fisher Scientific); analytical column, Dionex CarboPac PA210-Fast-4 μm analytical column (2.0 × 150 mm, Thermo Fisher Scientific); column oven temperature, 35ºC; mobile phase flow rate, 0.15 mL/min; injection volume, 2.5 μL. Elution gradients are as follows: 0–12 min, 12 mM KOH; 12–12.1 min, 12–100 mM KOH; 12.1-20 min, 100 mM KOH; 20–20.1 min, 100–12 mM KOH; and 20.1–32 min, 12 mM KOH. To estimate the concentrations of glucose, fructose, maltose, sucrose, and isomaltose, standard glucose, fructose, maltose, sucrose, and isomaltose were used to make a standard curve.

Preparation of α-glucosidase solution Rat intestinal acetone powder (Sasaki Chemical, Kyoto, Japan) (0.5 g) in 5 mL of water was sonicated. The solution was centrifuged at 20,000 × *g* for 20 min at 4ºC. The supernatant was diluted with water to be 70 mg/mL.

Measurement of maltase, sucrase, and isomaltase activities The reaction was carried out as described previously [10, 11]. Briefly, 70 μL of 0.1 M phosphate buffer (pH 6.0), 10 μL of maltose or sucrose solution, and 10 μL of the wine, juice, vinegar drink, vinegar, or jam 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. After 2 min, the solution was boiled for 5 min to stop reaction. To 13.3 μL of the solution, 200 μL of the coloring solution in Glucose CII test was added for measurement of maltase activity. To 100 μL of the solution, 100 μL of the coloring solution in Glucose CII test was added for measurement of sucrase and isomaltase activity. The absorbance at 505 nm (A_{505}) was measured with an EnSight multimodal plate reader (PerkinElmer, Waltham, MA). The glucose concentrations were determined according to the calibration curves made in the presence of various concentrations of respective foods.

Measurement of glucoamylase activity For measurement of glucoamylase (starch hydrolase releasing β-glucose from the non-reducing ends of starch) activity (A) plus other α-glucosidase (aryl-glucosides hydrolase releasing β-glucose) activity (B), 50 μl of β-glucosidase in water, 50 μL of 4-nitrophenyl-β-maltoside (G2-β-PNP) in water, and 25 μL of wine, juice, vinegar drink, vinegar, or jam solution were mixed and preincubated at 37ºC for 10 min. For measurement of other α-glucosidase activity (B), 200 μL of PNPG in water and 25 μL of wine, juice, vinegar drink, vinegar, or jam solution were mixed and preincubated at 37ºC. After the pre-incubation, the reaction was started by adding 10 μL of α-glucosidase solution and incubated at 37ºC for 10 min. Then, the Na_2CO_3 solution (100 or 200 μL) was added to stop reaction. The A_{400} was measured with an EnSight, and the reaction rates for A+B and that for B were obtained, respectively. The reaction rate for A was calculated by reducing B from A+B.

Results and Discussion

Quantification of iminosugars in *M. australis* fruit foods

We applied five *M. australis* fruit foods to LC-MS/MS. Figure 1A–C show the MRM

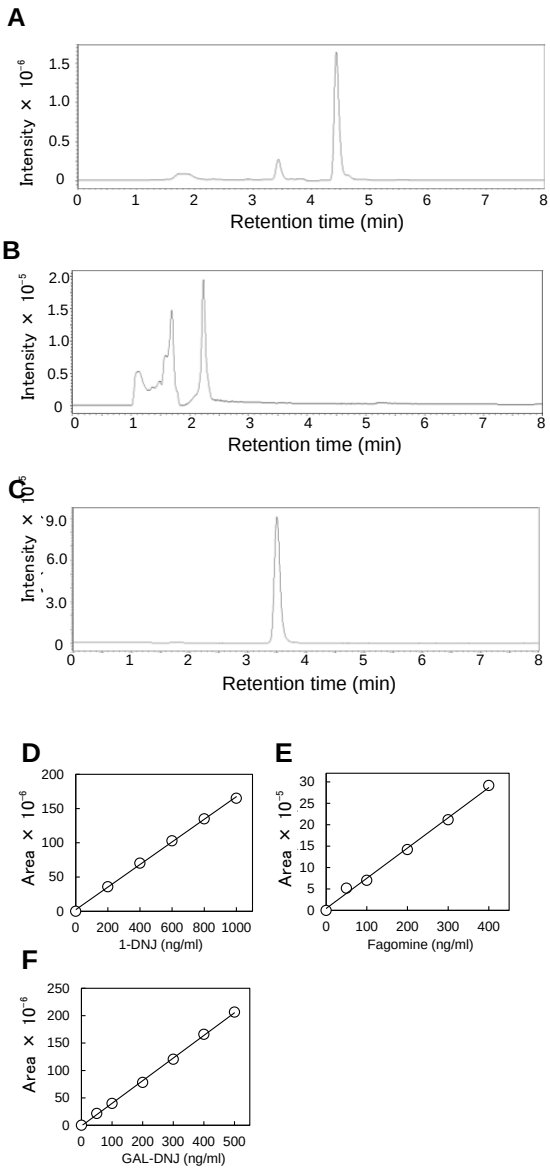

Fig. 1. Quantification of iminosugar. (A–C) MRM chromatogram of *M. australis* fruit wine. MRM chromatogram of the total of ions at *m/z* 69.20 $[M^+H^-95]^+$, 80.20 $[M^+H^-84]^+$, and 146.20 $[M^+H^-H_2O]^+$ for 1-DNJ (A), at 86.15 $[M^+H^-62]^+$, 112.15 $[M^+H^-2H_2O]^+$, and 130.15 $[M^+H^-H_2O]^+$ 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. (D–F) Calibration curve (0–1,000 ng/mL) of iminosugars. (D) 1-DNJ. (E) Fagomine. (F) GAL-DNJ.

chromatogram of wine. One peak at 4.4 min appeared for 1-DNJ (A), which was used for analysis. Three peaks at 1.6, 1.8, and 2.4 min, respectively, appeared for fagomine (B), from which the peak at 2.4 min was used for analysis since the peak for the standard fagomine was 2.4 min. One peak at 3.5 min appeared for GAL-DNJ (C), which was used for analysis. Similar patterns were obtained for juice, vinegar drink, vinegar, and jam (data not shown). Figure 1D–F show a calibration curve, which was made by applying various concentrations of standard 1-DNJ (D), fagomine (E), and GAL-DNJ (F). The peak instensity increased linearly with increasing concentration (0–1,000 ng/mL) of each iminosugar. Based on the calibration curves, the iminosugar concentrations in the *M. australis* fruit foods were calculated (Table 1). The GAL-DNJ concentration was highest (30.2–209 μg/mL) followed by 1-DNJ (1.76–32.5 μg/mL) and fagomine (0.058–0.290 μg/mL). Fagomine was detected in non-mulberry wines. As far as we know, there are no such reports.

Table 1. Concentrations of iminosugar in the *M. australis* and non-mulberry fruit foods.

	1-DNJ (μg/mL)	fagmoine (μg/mL)	Gal-DNJ (μg/mL)
<Mullberry>			
Wine	32.5	0.117	209
Juice	8.21	0.237	73.7
Vinegar drink	1.76	0.066	122
Vinegar	7.38	0.290	30.2
Jam	9.45	0.058	41.0
<Non-mulberry>			
Wine #1	< LOQ	0.0096	< LOQ
Wine #2	< LOQ	0.0091	< LOQ
Wine #3	< LOQ	0.070	< LOQ

Quantification of sugar in *M. australis* fruit foods

Recently, high-performance anion-exchange chromatography with pulsed amperometric

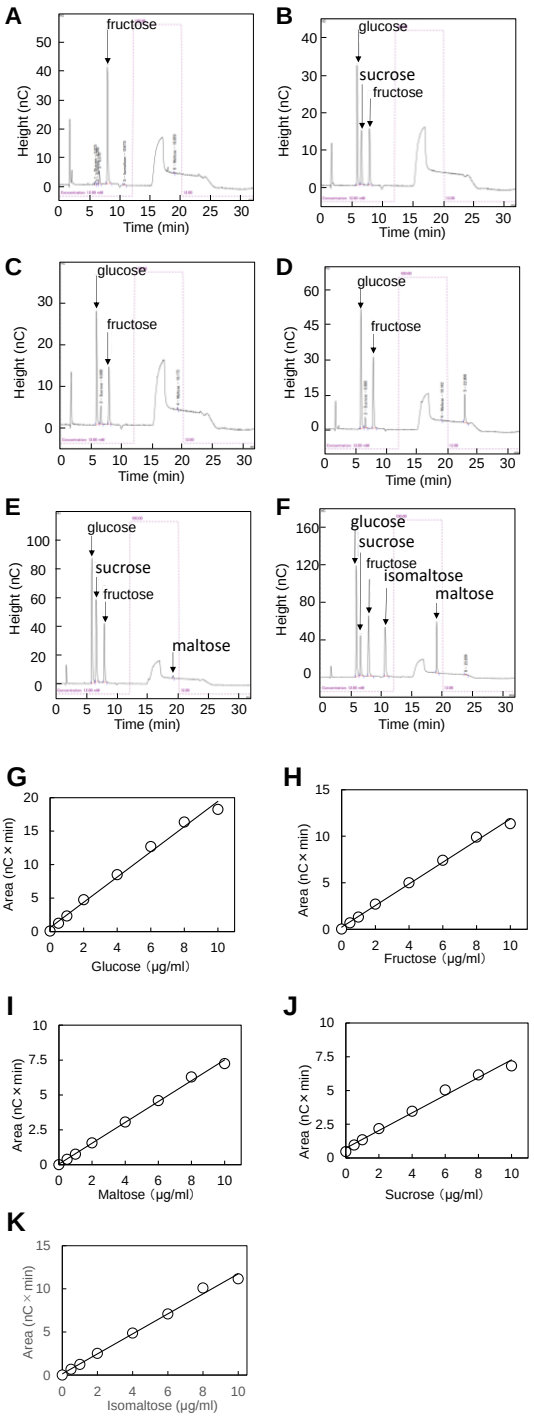

Fig. 2. HPAE-PAD. Analysis. (A–F) Elution patterns of *M. australis* fruit foods (wine (A), juice (B), vinegar drink (C), vinegar (D), and jam (E)) and the mixture of glucose, fructose, maltose, sucrose, and isomaltose (F) are shown. (G–K) Calibration curves (0–10 μg/mL) of glucose (G), fructose (H), maltose (I), sucrose (J) and isomaltose (K) are shown.

detection (HPAE-PAD) is applied to detect very low levels of carbohydrate products with high accuracy [15–17]. Compared to the enzyme-based oxidase method, HPAE-PAD is thought to be less affected by the contaminants. We applied five *M. australis* fruit foods to HPAE-PAD. Figure 2A–E show the elution pattern of wine (A), juice (B), vinegar drink (C), vinegar (D), and jam (E). Several peaks appeared in each food. Figure 2F shows the elution pattern of the mixture of standard glucose, fructose, maltose, sucrose, and isomaltose. The elution times of each sugar (5.9 min for glucose, 7.9 min for fructose, 19.1 min for maltose, 6.6 min for sucrose, and 10.6 min for isomaltose) were determined according to the elution patterns of each sugar alone (data not shown). Figure 2G–K shows calibration curves, which were made by applying various concentrations of standard glucose, fructose, maltose, sucrose, and isomaltose. The peak intensity increased linearly with increasing concentration (0–10 μg/mL) of each sugar. Based on the calibration curves, the sugar concentrations in the *M. australis* fruit foods were calculated (Table 2). The glucose concentration of wine was 0 mg/mL while those of juice, vinegar drink, vinegar, and jam were 13.9–39.7 mg/mL.

Table 2. Concentrations of sugar in the *Morus australis* fruit foods.

	glucose (mg/mL)	fructose (mg/mL)	Maltose (mg/mL)
Wine	0	22.8	< LOQ
Juice	39.7	37.3	< LOQ
Vinegar drink	33.3	33.9	< LOQ
Vinegar	13.9	16.5	< LOQ
Jam	25.9	22.7	< LOQ
	sucrose (mg/mL)	isomaltose (mg/mL)	
Wine	< LOQ	< LOQ	< LOQ
Juice	41.3	< LOQ	< LOQ
Vinegar drink	< LOQ	< LOQ	< LOQ
Vinegar	< LOQ	< LOQ	< LOQ
Jam	61.0	< LOQ	< LOQ

We previously reported the glucose concentrations of the *M. australis* fruits were 123–262 mg/g [13]. This suggested that in the wine, glucose was consumed completely during fermentation.

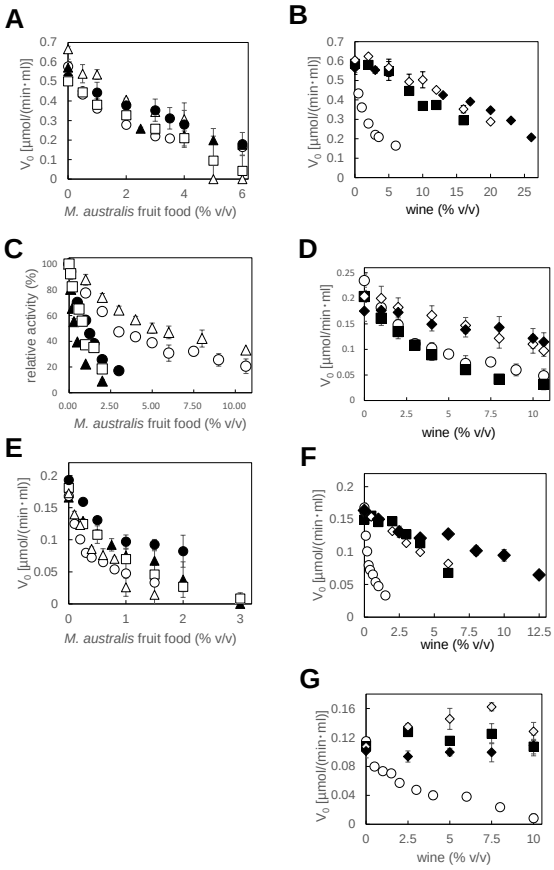

Fig. 3. Dependence on the food concentrations of the reaction rate for the hydrolysis by rat intestinal acetone powder. The reaction was carried out with 3.5 mg/mL (A, B, G) or 7.0 mg/ml (C–F) rat intentional acetone powder solution and 50 mM maltose (A, B), 15 mM G2-β-PNP (C, D), 100 mM sucrose (E, F) of 50 mM isomaltose (G) in the presence of varying concentrations of *M. australis* fruit wine (open circle), juice (open triangle), vinegar drink (closed circle), vinegar (closed triangle), or jam (open square) (A, C, E) or *M. australis* fruit wine (open circle), non-mulberry fruit wine #1 (closed square), #2 (open diamond), or #3 (closed diamond) (B, D, F, G) at pH 6.0 at 37ºC. Error bars indicate SD values. The average of triplicate determination is shown.

Inhibition of α-glucosidase by *M. australis* or non-mulberry fruit food

We examined the inhibitory effects of the five *M. australis* fruit foods and three non-mulberry reaction rates in the hydrolysis of maltose (A, B), G2-β-PNP (C, D), sucrose (E, F), and isomaltose (G) in the presence of varying concentrations of food at pH 6.0 at 37ºC. The initial reaction rates decreased with increasing the concentration of food. Based on the data, the IC_{50} values were calculated (Table 3). The IC_{50} values of *M. australis* fruit foods were 1.9–4.4 % v/v for maltase, 0.37-5.0% for glucoamylase, and 0.28–1.2% for sucrase. The IC_{50} values for isomaltase was 2.0% for *M. australis* fruit wine, but were not obtained for other foods. The IC_{50} values of non-mulberry wines were 16–24% for maltase which are 8.4–13 fold higher than that of *M. australis* fruit wine (1.9%), 3.4–10% for glucoamylase which are 1.2–3.7 fold higher than that of *M. australis* fruit wine (2.8%), and 5.7–11% for sucrase which are 20–39 fold higher than that of *M. australis* fruit wine (0.28%). This suggested that the difference might mainly be due to that *M. australis* fruit wine contained 1-DNJ while non-mulberry wines did not (Table 1). The difference in IC_{50} values between *M. australis* fruit wine and non-mulberry wine for glucoamylase were smaller than for maltase and sucrase (Table 3). This suggested that not only 1-DNJ but also other components, presumably polyphenols, are involved in the inhibition of glucoamylase considerably.

According to the 1-DNJ concentration in Table 1 (A) or glucose concentration in Table 2 (B) and the IC_{50} values for maltose in Table 3 (C) of the *M. australis* fruit foods, we calculated the 1-DNJ concentration (A × C) or glucose concentration (B × C) of the food solution thought to exhibit 50% inhibition for maltase (Table 4). We previously reported that the IC_{50} values for maltose of 1-DNJ and glucose were 0.70 μg/mL and 0.69 mg/mL, respectively [11]. The 1-DNJ concentration in the wine that were thought to exhibit 50% inhibition for maltase was calculated to be 0.62 μg/mL, which is almost equal to the IC_{50} values for maltase of 1-DNJ (0.70 μg/mL), suggesting that 1-DNJ is a main inhibitory for maltase. In other four foods, the 1-DNJ and glucose concentrations that were thought to exhibit 50% inhibition for maltase were calculated to be 0.077–0.30 μg/mL and 0.32–1.5 mg/mL, respectively., which were comparable to the IC_{50} values for maltase of 1-DNJ and glucose (0.70 μg/mL and 0.69 mg/mL, respectively). This suggested that 1-DNJ is a main inhibitor for α-glucosidase in these foods, and glucose is involved in the inhibition except for wine.

Table 3. IC_{50} values of the *Morus australis* and non-mulberry fruit foods.

	maltase (%, v/v)	glucoamyla-se (%, v/v)	sucrase (%, v/v)
<Mulberry>			
Wine	1.9	2.8	0.28
Juice	3.3	5.0	0.40
Vinegar drink	4.4	1.2	1.2
Vinegar	2.3	0.37	0.88
Jam	3.2	0.87	0.75
<Non-mulberry>			
Wine #1	16	3.4	5.7
Wine #2	19	10	6.0
Wine #3	24	ND	11

Table 4. Calculated 1-DNJ and glucose concentrations in the food solution in which the concentration is equal to IC_{50} values for maltase.

	1-DNJ (μg/mL)	Glucose (mg/mL)
Wine	0.62	< LOQ
Juice	0.27	1.3
Vinegar drink	0.077	1.5
Vinegar	0.17	0.32
Jam	0.30	0.83

Our results also suggested that 1-DNJ was not much degraded during production process. According to the manufacturer's information, the production processes of these five foods contain heat treatment at 70–80ºC for 1–2 h. To address this issue, we examined the thermostabilities of iminosugars. 1-DNJ, fagomine, and GAl-DNJ were incubated at 80ºC for 0–24 h followed by LC-MS/MS. The peak area were stable, indicating that each iminosugar is stable at 80ºC. This suggested that it does not degrade during the production process.

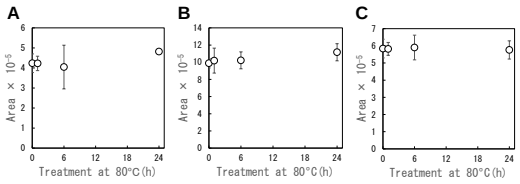

Fig. 4. Stability of iminosugar. 1-DNJ (A), fagomine (B), and GAL-DNJ (C) were incubated at 80ºC for indicated times, and then applied to LC-MS/MS. The peak area in LC-MS/MS is shown. Error bars indicate SD values. The average of triplicate determination is shown.

Conclusion

M. australis fruit foods, wine, juice, vinegar drink, vinegar, and jam, contain 1-DNJ and inhibits α-glucosidase activity. Our results suggest that *M. australis* fruit foods are healthy sweeter.

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