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Food-Grade Mulberry Powder Enriched with 1-Deoxynojirimycin Suppresses the Elevation of Postprandial Blood Glucose in Humans

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Mulberry 1-deoxynojirimycin (DNJ), a potent glucosidase inhibitor, has been hypothesized to be beneficial for the suppression of abnormally high blood glucose levels and thereby prevention of diabetes mellitus. However, DNJ contents in commercial mulberry products were as low as about 0.1% (100 mg/100 g of dry product), implying that the bioavailability of DNJ might not be expected. We carried out studies in two directions: (1) production of food-grade mulberry powder containing a maximally high DNJ content; (2) determination of the optimal dose of the DNJ-enriched powder for the suppression of the postprandial blood glucose through clinical trials. The following method was used: (1) DNJ concentrations in mulberry leaves from different cultivars, harvest seasons, and leaf locations were determined using hydrophilic interaction chromatography with evaporative light scattering detection. (2) Healthy volunteers received 0, 0.4, 0.8, and 1.2 g of DNJ-enriched powder (corresponding to 0, 6, 12, and 18 mg of DNJ, respectively), followed by 50 g of sucrose. Before and 30−180 min after the DNJ/sucrose administration, plasma glucose and insulin were determined. The following results were obtained: (1) Young mulberry leaves taken from the top part of the branches in summer contained the highest amount of DNJ. After optimization of the harvesting and drying processes for young mulberry leaves (*Morus alba* L. var. Shin ichinose), DNJ-enriched powder (1.5%) was produced. (2) A human study indicated that the single oral administration of 0.8 and 1.2 g of DNJ-enriched powder significantly suppressed the elevation of postprandial blood glucose and secretion of insulin, revealing the physiological impact of mulberry DNJ (effective dose and efficacy in humans). This study suggests that the newly developed DNJ-enriched powder can be used as a dietary supplement for preventing diabetes mellitus.

KEYWORDS: 1-Deoxynojirimycin; HILIC-ELSD; mulberry leaves; *Morus* spp.; diabetes prevention

INTRODUCTION

In the past three decades, there has been a continued interest in iminosugars (also termed azasugars) due to their high potency as glycosidase inhibitors (*1*−*4*). Their physiological activity in the digestive tract (i.e., a delay of the intestinal digestion and absorption of carbohydrates) has been investigated. Some iminosugars (i.e., the antidiabetic drug miglitol) showed a significant hypoglycemic effect (*5*−*7*), which provides grounds for a more comprehensive study of the usability of naturally occurring iminosugars in plant foods.

Mulberry leaves (Moraceae) rich in iminosugars such as the glucose analogue 1-deoxynojirimycin (DNJ; **Figure 1**), *N*-methyl-DNJ, and 2-*O*-α-D-galactopyranosyl-DNJ, DNJ being the most abundant and accounting for 50% of the mulberry iminosugars (*8*). Since DNJ is believed to be the most bioactive agent (α-glucosidase inhibitor), dietary mulberry DNJ might be beneficial for suppressing abnormally high blood glucose levels, thereby helping prevent diabetes mellitus (*9*−*12*). At present, various food-grade mulberry products (i.e., teas, powders, and tablets) have been made commercially available in Japan and many other countries. These products suggest the bioavailability

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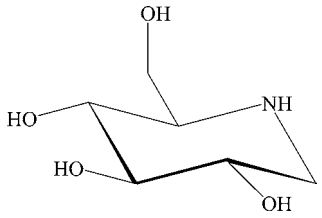

Figure 1. Chemical structure of DNJ.

of DNJ; however, their DNJ contents and efficacy have never been specified.

We previously developed a method for determining DNJ by hydrophilic interaction chromatography with evaporative light scattering detection (HILIC-ELSD) (*13*). Using HILIC-ELSD, we confirmed that the DNJ contents in mulberry products are as low as about 0.1% ((100 mg/100 g of dry weight)/product) and that there are some products with a trace amount of DNJ (less than 0.05%). Since the estimated effective dose (more than 10 mg of DNJ/60 kg human) cannot be provided by current commercial mulberry products due to their low DNJ content, the development of DNJ-enriched products is highly desired.

In this study, we carried out studies in two directions: (1) production of food-grade mulberry powder containing a maximally high DNJ content conforming with cultivar differences, harvesting season, mulberry leaf location, and optimization of harvesting and drying processes; (2) determination of the optimal dose of the DNJ-enriched powder for the suppression of the postprandial blood glucose through clinical trials.

MATERIALS AND METHODS

Chemicals. Standard DNJ was purchased from Wako Pure Chemical (Osaka, Japan). Acetonitrile, ethanol, and distilled water were obtained from Kanto Chemical (Tokyo, Japan). Ammonium acetate was obtained from Sigma (St. Louis, MO). All other reagents used were of analytical grade.

HILIC-ELSD. The chromatography system consisted of a Shimadzu LC-10AD pump (Kyoto, Japan), a Shimadzu DGU-14A degasser, a Shimadzu CTO-10A column oven, and a Reodyne 7125 injector (Cotati, CA). A TSKgel Amide-80 column (4.6 × 250 mm; Tosoh, Tokyo, Japan) was used as the HILIC column. The separation was performed using a mixture of acetonitrile and distilled water (72:28, v/v; containing 6.5 mmol/L ammonium acetate, pH 5.5). The flow rate was adjusted to 1.0 mL/min, and the column temperature was maintained at 40 °C. The eluent was split at the postcolumn. One of the split eluents (flow rate 0.95 mL/min) was sent to an SEDEX 55 evaporative light scattering detector (Sedere, Alfortville, France). The temperature of the drift tube was set at 55 °C, nitrogen gas was used at a pressure of 1200 hPa, and the gain was set at 8. The DNJ peak area was registered using a Shimadzu C-R6A Chromatopac integrator. The other split eluent (flow rate 0.05 mL/min) was sent to a Mariner electrospray ionization time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA). The mass spectrometer was used in the positive ion measurement mode with a spray voltage of 2900 V, a nozzle potential of 220 V, and a nozzle temperature of 140 °C. The flow rate of the nebulizer gas was 0.3 mL/min. Full scan spectra were obtained by scanning masses between *m*/*z* 100 and *m*/*z* 2000 at 3 s/scan.

Plant Materials. Thirty-four varieties of mulberry leaves (*Morus alba* L. var. Aobanezumi, Hachinose, Hayatesakari, Hinosakari, Ichinose, Kairyou nezumigaeshi, Kinuyutaka, Kokuso 21, Minamisakari, Mitsusakari, Natsunobori, Ooyutaka, Shin ichinose, Shuukakuichi, Tagowase, Tomieisou, and Tsuruta; *Morus bombycis* Koidz. var. Akagi, Aokitakasuke, Chikuma ooha, Fushimagari, Ichibei, Kairyou akita, Matsumoto 1, Mitsushigeri, Ruinashi, Shin kenmochi, and Yukiasahi; *Morus latifolia* Poir. var. Atsubamidori, Azumiguwa, Mitsuminami, Rosou, Tachimidori, and Wasemidori), grown in the field at Fukushima Agricultural Technology Centre (Date, Fukushima, Japan), were used in this study.

On June 15, 2004, nine branches (approximately 70 cm in length) were collected from three trees of each cultivar. Similarly, on June 15, July 15, Aug 12, and Sept 16, 2004, nine branches were collected from *M. alba* L. var. Ichinose and Kairyou nezumigaeshi. The nine branches collected on June 15 were cut into two pieces (top and bottom pieces, each approximately 35 cm). Also, the nine branches collected on July 15, Aug 12, and Sept 16 were cut equally into three pieces (top, middle, and bottom pieces, approximately 70 cm in length). Following that, mulberry leaves were collected from the branches. The leaves were cleaned with water, lyophilized, and disintegrated.

Quantification of Mulberry DNJ. Mulberry DNJ was determined using HILIC-ELSD as previously reported by us (*13*). Briefly, the disintegrated mulberry leaves (0.1 g) were mixed with 1.0 mL of a mixture of acetonitrile and water (50:50, v/v; containing 6.5 mmol/L ammonium acetate, pH 5.5) in a microtube. After sonication for 1 min, the mixture was centrifuged at 15000*g* for 5 min. The supernatant was filtered through a PTFE filter (0.45 μm pore size; Advantec, Tokyo, Japan), and a 10 μL aliquot was subjected to HILIC-ELSD. The DNJ concentration in the leaves was calculated using the calibration curve equation of standard DNJ. Quantification was performed within 3 months after leaf sampling.

Optimization of the Harvesting and Drying Processes for Mulberry Leaves. For the harvesting process, a method involving a plucking machine, considered an effective way to collect young leaves, was used. For the drying process, 100 g of fresh mulberry leaves (from a whole branch of *M. alba* L. var. Kairyou nezumigaeshi) was separately treated with three different drying processes: hot-air drying (80 °C, 24 h; Soyokaze, Isuzu, Tokyo, Japan), low-temperature dehumidification (20 °C, 48 h; IHR-06-4, Inabaya Reinetsu Sangyo, Tokyo, Japan), and lyophilization (−40 °C, 48 h; Takara Seisakusho, Tokyo, Japan). The DNJ content of the different dried leaves was determined using HILIC-ELSD.

Mulberry Powder Enriched with DNJ. Young mulberry leaves (480 kg, *M. alba* L. var. Shin ichinose) were collected from the top part of the branches in August 2005 using a plucking machine (V8X2, Ochiai Cutlery Manufacturing, Kikukawa, Japan). The young mulberry leaves were subjected to the hot-air dryer (80 °C, 24 h). The dried leaves (100 kg) were disintegrated and mixed with 2000 L of a mixture of ethanol and water (20:80, v/v). After filtration, the extract was concentrated and lyophilized to a powder (15 kg). The DNJ content in the powder was checked using HILIC-ELSD.

Human Study. To determine the effective dose of DNJ-enriched powder, a single oral administration study was conducted. Twenty-four healthy volunteers (age, 25.3 ± 0.7 years; body mass index, 20.9 ± 0.4 kg/m^2) participated. All subjects were generally healthy as demonstrated by their medical history and a physical examination. After fasting for 12 h, the subjects were randomly divided into four groups of six individuals. Each group received 0 (placebo), 0.4, 0.8, or 1.2 g of DNJ-enriched powder (corresponding to 0, 6, 12, or 18 mg of DNJ, respectively), followed by 50 g of sucrose dissolved in 100 mL of water. Blood samples (10 mL) were collected before DNJ/sucrose intake and 30, 60, 90, 120, 150, and 180 min after the administration.

To evaluate the long-term effect of DNJ-enriched powder on biochemical parameters, a daily administration study was conducted. Twelve healthy volunteers (age, 24.7 ± 1.0 years; body mass index, 21.3 ± 0.6 kg/m^2) participated. The subjects were randomly divided into two groups and received 0 (placebo) or 1.2 g of DNJ-enriched powder before every meal (0 or 3.6 g/day) for 38 days. At days 0, 24, and 38 of DNJ-enriched powder administration, 12 h fasting blood was collected.

The blood samples were subjected to centrifugation at 1000*g* for 10 min at 4 °C to prepare plasma. The plasma glucose was determined by the glucose oxidase method using Cica Liquid GLU (Kanto Chemical, Tokyo, Japan). Plasma insulin was measured by enzyme immunoassay using LS reagent Eiken Insulin (Eiken Chemical, Tokyo, Japan). Other plasma biochemical parameters (total cholesterol, HDL cholesterol, and triglyceride) and hemoglobin A_{1C} (HbA_{1C}) were determined by using commercial kits. The study was approved by the Ethics Committee at Nippon Medical School (Tokyo, Japan), and was conducted in compliance with the Helsinki Declaration. All subjects had given written informed consent.

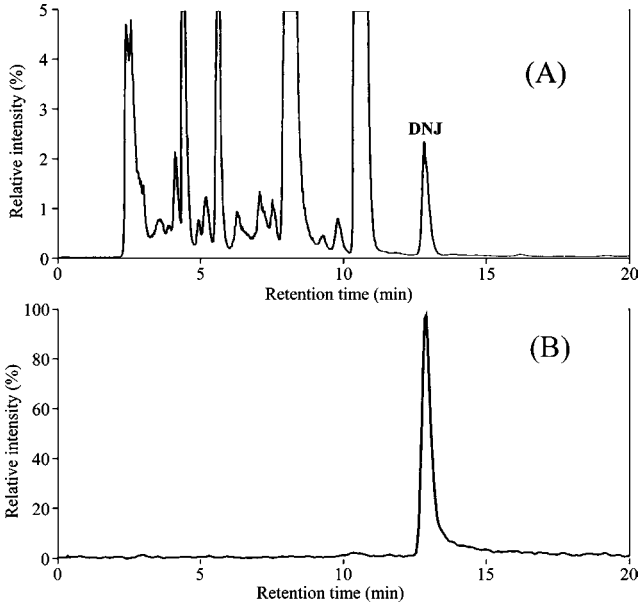

Figure 2. HILIC-ELSD–MS analysis of mulberry DNJ: (**A**) ELSD chromatogram, (**B**) single ion plot of the mass corresponding to the $[M + H]^+$ ion of DNJ (*m*/*z* 164.1). Leaves were collected from a whole branch of *M. alba* L. var. Kairyou nezumigaeshi, a standard cultivar in Japan, and then analyzed by HILIC-ELSD–MS under the following conditions: column, TSKgel Amide-80 (4.6 × 250 mm); mobile phase, a mixture of acetonitrile and distilled water (72:28, v/v; containing 6.5 mmol/L ammonium acetate, pH 5.5); flow rate, 1 mL/min; column temperature, 40 °C.

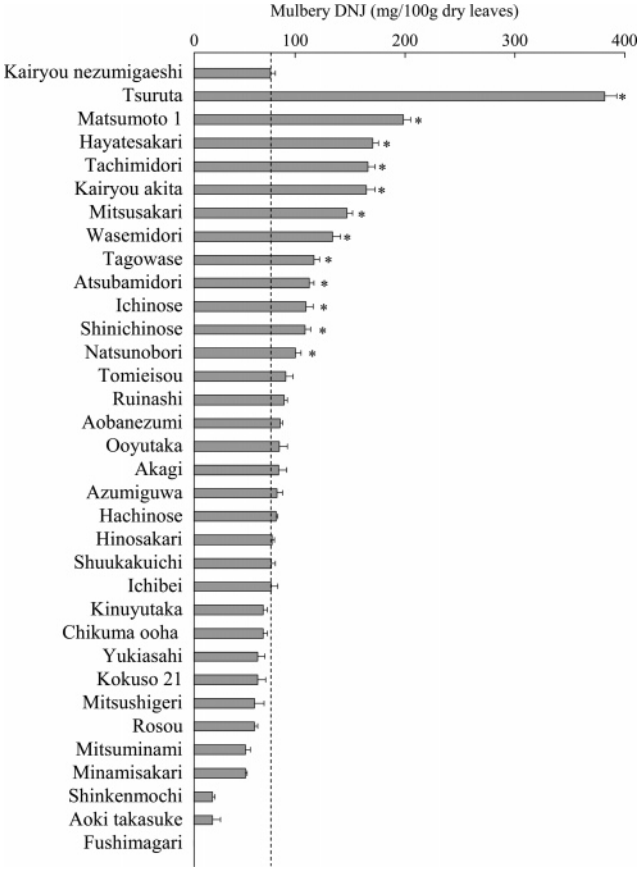

Figure 3. DNJ concentrations in mulberry leaves from whole branches of different cultivars. The leaves were collected from whole branches of the different cultivars on June 15, 2004. The DNJ in these leaves was measured using HILIC-ELSD. The data represent the mean ± SEM ($n = 3$). An asterisk indicates $P < 0.05$ vs *M. alba* L. var. Kairyou nezumigaeshi, a standard cultivar in Japan.

Statistical Analysis. The data were expressed as means ± SEM. The results were subjected to ANOVA, and differences among the means were determined using Bonferroni's test. Statistical calculation was carried out using ystat 2000, an Excel statistical program file (Igaku Tosho Shuppan, Tokyo, Japan). Differences with $P < 0.05$ were considered significant.

RESULTS

Suitable Mulberry Sources and Methods for Producing DNJ-Enriched Powder. Figure 2**A** presents a typical ELSD chromatogram taken after a mulberry leaf extract (*M. alba* L. var. Kairyou nezumigaeshi, a standard cultivar in Japan) was subjected to HILIC-ELSD. A peak appeared at a retention time of 12.8 min and was identified as DNJ on the basis of single ion plot MS analysis (**Figure 2B**). **Figure 3** presents DNJ concentrations in the mulberry leaves obtained from whole branches of the different cultivars on July 15, 2004. Relatively high DNJ concentrations were found in some cultivars (i.e., *M. alba* L. var. Tsuruta, Matsumoto 1, Hayatesakari, Tachimidori, Kairyou akita, Mitsusakari, Wasemidori, Tagowase, Atsubamidori, Shin ichinose, and Natsunobori). The concentrations were about 1.3−5.3 times the level of DNJ found in a general mulberry leaf (*M. alba* L. var. Kairyou nezumigaeshi). As depicted in **Figure 4**, the harvest season and the region of mulberry leaves (*M. alba* L. var. Kairyou nezumigaeshi) were closely related to the DNJ content; that is, young mulberry leaves taken from the top part of the branches in the summer were enriched in DNJ. A similar tendency was found in the other cultivars (*M. alba* L. var. Ichinose, data not shown). Concerning the processes (i.e., the harvesting and drying processes for mulberry leaves), we found that a plucking machine was highly useful for a selective collection of the young mulberry leaves (data not shown). In the drying step, we chose hot-air drying because of its cost-effectiveness since the three processes (hot-air drying, low-temperature dehumidification, and lyophilization) did not cause serious DNJ degradation (data not shown).

Considering the results (**Figures 3** and **4**) and current usability of mulberry cultivars in Japan (i.e., productivity and yield), we selected young leaves from the top part of branches of *M. alba* L. var. Shin ichinose for large-scale production of DNJ-enriched powder. DNJ was extracted from the dried leaves of the top part of branches of *M. alba* L. var. Shin ichinose using a mixture of ethanol and water (20:80, v/v). Food-grade DNJ-enriched powder (1.5%) could be produced by lyophilizing the extract to a powder. This DNJ-enriched powder contained DNJ at a level about 15 times higher than that of general commercial mulberry products (i.e., teas, powders, and tablets).

Therapeutic Effect of Mulberry Powder Enriched with DNJ. Using the developed DNJ-enriched mulberry powder, a human study was conducted to investigate the change in the postprandial blood glucose level. As seen in **Figure 5A**, intake of DNJ-enriched powder decreased the elevated plasma glucose level in the subjects. The effective dose of DNJ-enriched powder was observed in the groups of 0.8 and 1.2 g of DNJ-enriched powder (corresponding to 12 and 18 mg of DNJ). Similarly, subjects taking DNJ-enriched powder showed a decrease in plasma insulin secretion compared to subjects taking a placebo (**Figure 5B**). Since the suppression of both plasma glucose and insulin is the characteristic feature of an α-glucosidase inhibitor, DNJ in the mulberry powder would act as an intestinal α-glucosidase inhibitor. As shown in **Figure 6**, daily intake of

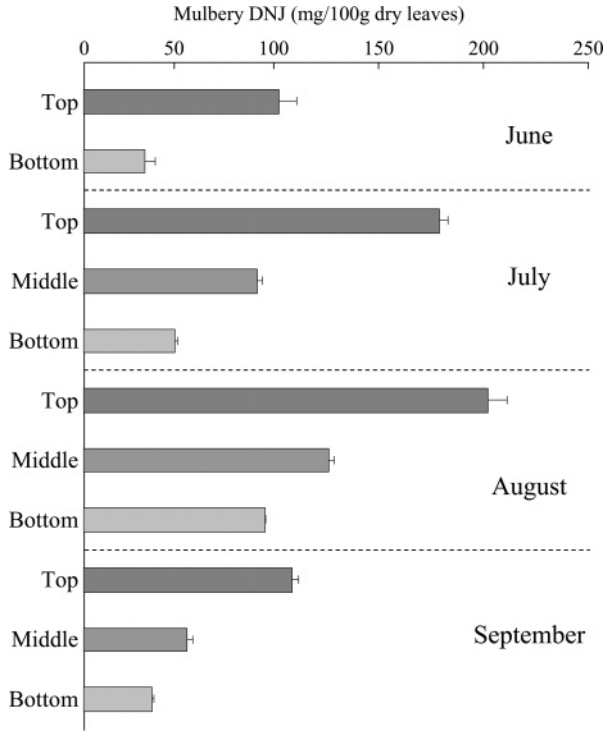

Figure 4. DNJ concentrations in mulberry leaves from different harvest seasons and harvest regions. Leaves (*M. alba* L. var. Kairyou nezumigaeshi) were collected from the top, middle, and bottom parts of branches cut on June 15, July 15, Aug 12, and Sept 16, 2004. The DNJ in these leaves was measured using HILIC-ELSD. The data represent the mean ± SEM ($n = 3$).

DNJ-enriched powder did not cause hypoglycemia (low blood sugar) as well as abnormal lipid profiles (i.e., high cholesterol).

DISCUSSION

Glycosidases are involved in a wide range of biological metabolism processes, such as digestion, lysosomal catabolism of glycoconjugates, biosynthesis of glycoproteins, and endoplasmic reticulum quality control (*14*). Modification of these processes by dietary foods and drugs is of interest from a therapeutic point of view. Iminosugars (azasugars), including DNJ, are an important class of glycosidase inhibitors that have been receiving considerable attention as potential therapeutic agents (i.e., antidiabetics, antiobesities, and antivirals) (*15*). Among the iminosugars, miglitol (Glyset) has been approved as a drug (a second-generation α-glucosidase inhibitor) to treat type 2 diabetes (*16*, *17*). *N*-Butyl-DNJ (Zavesca) has also been used as a drug for patients with type 1 Gaucher disease (*18*). Despite DNJ's excellent α-glucosidase inhibitory activity in vitro, its efficacy in vivo was reported to be rather moderate (*19*). We therefore consider DNJ to be suitable for use as a "functional food" instead of as a drug.

Since mulberry leaves contain DNJ (*13*), mulberry leaf products (i.e., teas, powders, and tablets) have been commercialized as health food. However, in our previous experiments (*13*), we found that the DNJ content of commercial mulberry products was as low as 0.1% (100 mg/100 g of dry product) and that there were some products with only a trace amount of DNJ (less than 0.05%). According to a recent animal study by Miyahara et al. (*11*), administration of the ethanol extract from mulberry leaves (EM) to rats 30 min before carbohydrate intake dose-dependently suppressed the postprandial rise of blood glucose. The effective doses of EM were, however, rather large (0.1−0.4 g/kg of rat), equivalent to 6−24 g of EM/60 kg human. The dose (6−24 g of EM/60 kg human) corresponds to 6−24 mg of DNJ/60 kg human, assuming the EM contains 0.1% DNJ. However, the dose of 6−24 mg of DNJ/60 kg human cannot be provided by currently available mulberry products due to their low DNJ content (around 0.1%). Therefore, development of DNJ-enriched nutraceutical products is desired.

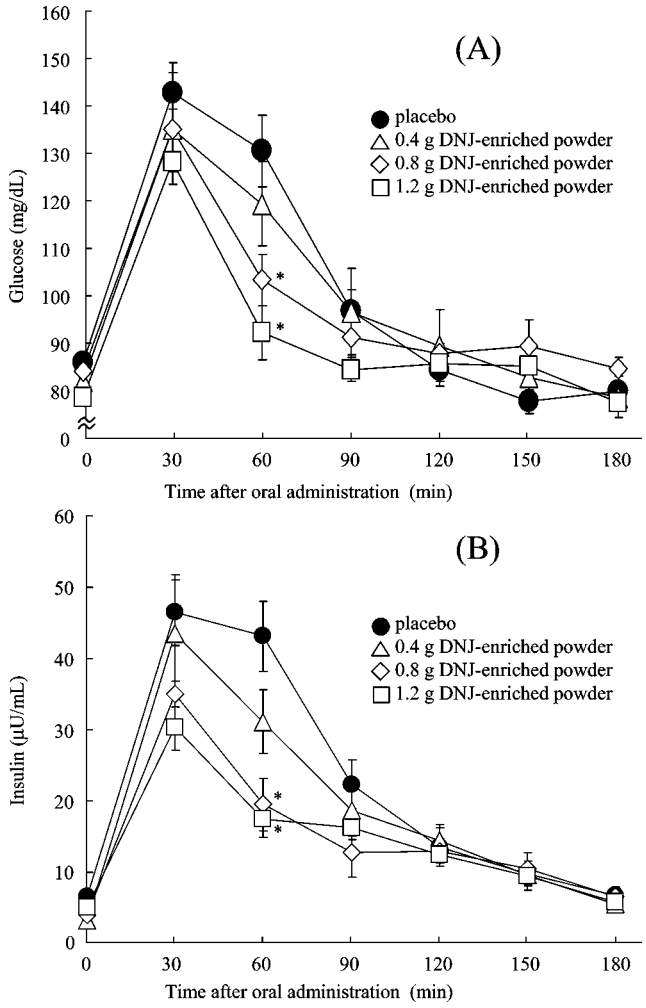

Figure 5. Effects of single oral administration of the mulberry powder enriched with DNJ (1.5%) on plasma glucose (**A**) and insulin (**B**) levels. After fasting for 12 h, healthy volunteers were orally administered 0 (placebo), 0.4, 0.8, and 1.2 g of DNJ-enriched mulberry powder (0, 6, 12, 18 mg, of DNJ, respectively), followed by 50 g of sucrose in water. Blood samples were collected before intake and 30, 60, 90, 120, 150, and 180 min after the administration. The plasma glucose and insulin were determined. The data represent the mean ± SEM ($n = 6$). An asterisk indicates $P < 0.05$ vs the placebo.

In this study, we first demonstrated that young mulberry leaves taken from the top part of the branches in summer are rich in DNJ. The reason may be related to the biosynthesis of DNJ in plants. According to a postulated DNJ biosynthesis (based on data for dayflower, *Commelina communis*) (*20*), the first step is the C1 imination of glucose, followed by its reduction and oxidation. Then C1/C5 cyclization occurs to form nonojirimycin. The nojirimycin is finally dehydrated and reduced to DNJ. It is therefore likely that young mulberry leaves have high enzymatic activity related to imination, thereby producing a large amount of DNJ. Defensive activities of mulberry DNJ against herbivorous insects have also been

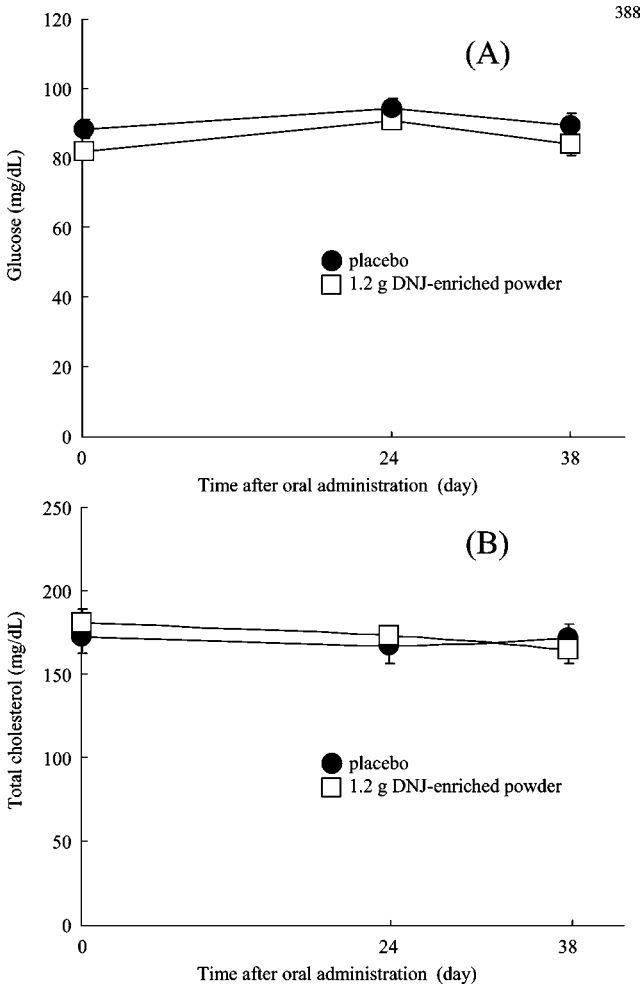

Figure 6. Long-term effects of the mulberry powder enriched with DNJ (1.5%) on plasma glucose (**A**) and total cholesterol (**B**) levels. The subjects received 0 (placebo) or 1.2 g of DNJ-enriched powder (18 mg of DNJ) before every meal for 38 days. At days 0, 24, and 38 of administration, 12 h fasting blood was collected. The plasma glucose and total cholesterol were determined. The data represent the mean ± SEM ($n = 6$).

reported (*21*). Young mulberry leaves may produce a high amount of DNJ because insects prefer young leaves.

In this study, when young mulberry leaves (*M. alba* L. var. Shin ichinose) were used, mulberry powder enriched with DNJ (1.5%) was produced. In the manufacturing process, hot-air drying was the most cost-effective drying process to avoid serious DNJ degradation. Besides *M. alba* L. var. Shin ichinose, some other cultivars (e.g., Tsuruta and Hayatesakari) also contained a high DNJ concentration. However, these cultivars (Tsuruta and Hayatesakari) are rarely cultivated in Japan. If Tsuruta (containing the highest level of DNJ) is used as the source, DNJ-enriched powder (more than 1.5% DNJ) can be produced.

We confirmed that the DNJ-enriched powder significantly suppressed the elevation of postprandial blood glucose and secretion of insulin (**Figure 5**) and the long-term administration of the DNJ-enriched powder did not cause hypoglycemia (**Figure 6**). To further confirm the efficacy of the DNJ-enriched powder, we are now conducting another clinical study. We have found that single administration of DNJ-enriched powder to borderline diabetic subjects suppressed the elevation of postprandial blood glucose. The administration of the DNJ-enriched powder has not caused any changes in the levels of blood markers (e.g., total protein, ALP, AST, ALT, LDH, γ-GTP, and T-Bil). These preliminary results suggest the safety of the DNJ-enriched powder; obviously future work will be required to conclude the safety of the DNJ-enriched powder in long-term use.

Although many reports have been published regarding the bioavailability of DNJ, there are no concrete, accurate data for the metabolic fate of DNJ. Faber et al. (*22*) studied the pharmacokinetics of 1-deoxymannojirimycin (DMJ) after intravenous administration of radiolabeled DMJ to rats. They found that the DMJ showed a rapid biphasic plasma disappearance. After 120 min of administration, 52% of the administrated DMJ was detected in unchanged form in the urine, whereas only 4.9% of the dose was excreted into bile. A small amount of DMJ was detected in the liver (2.1%), kidney (1.1%), small intestine (0.9%), stomach (0.6%), and heart (0.1%). These results suggested that iminosugars, including DNJ, may be rapidly eliminated from the body in a nonmetabolized form by renal excretion without any side effects. Mulberry powder enriched with DNJ, produced in this study, represents a powerful tool for the study of absorption and metabolism of DNJ in humans.

HILIC-ELSD, HILIC−MS, and HILIC−MS/MS analysis revealed that the DNJ-enriched mulberry powder contained DNJ and the other minor iminosugars (*N*-methyl-DNJ, 2-*O*-α-D-galactopyranosyl-DNJ, fagomine, and 1,4-dideoxy-1,4-imino-D-arabinitol) (*8*). Since the in vitro α-glucosidase (maltase and sucrase) inhibitory activity of DNJ which was evaluated by the rat intestine model was superior (4−138-fold) to that of *N*-methyl-DNJ, 2-*O*-α-D-galactopyranosyl-DNJ, fagomine, and 1,4-dideoxy-1,4-imino-D-arabinitol (*8*), the lowering effect of the blood-sugar level (**Figure 5**) would be mainly ascribed to DNJ.

In conclusion, mulberry powder enriched with DNJ (1.5%) could be obtained when young mulberry leaves (*M. alba* L. var. Shin ichinose) were used. Administration of DNJ-enriched powder to humans suppressed the rise of postprandial blood glucose. Although DNJ bioavailability in animals has been studied, we first revealed the human physiological impact of mulberry DNJ (effective dose and efficacy in humans). Current scientific evidence demonstrates that the risk of morbidity and mortality for diabetic patients can be diminished by aggressive treatment with diet, exercise, and pharmacological approaches to achieve better control of the blood glucose level. Dietary intake of the presently developed DNJ-enriched powder can inhibit all or some intestinal disaccharidases, which regulate the absorption of carbohydrates. Therefore, DNJ-enriched products are feasible for therapeutic use in oral treatment of non-insulin-dependent diabetes mellitus (type 2 diabetes). This possibility is now being investigated in clinical studies to obtain approval for Food for Specified Health Use (FOSHU) status in Japan.

ABBREVIATIONS USED

ALP, alkaline phosphatase; ALT, alanin aminotrasferase; AST, aspartate aminotransferase; DMJ, 1-deoxymannojirimycin; DNJ, 1-deoxynojirimycin; EM, ethanol extract from mulberry leaves; γ-GTP, γ-glutamyl transpeptidase; HILIC-ELSD, hydrophilic interaction chromatography with evaporative light scattering detection; LDH, lactate dehydrogenase; MS, mass spectrometry; T-Bil, total bilirubin.

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