Blood glycemia-modulating effects of melanian snail protein hydrolysates in mice with type II diabetes

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Abstract. Freshwater animal proteins have long been used as nutrient supplements. In this study, melanian snail (*Semisulcospira libertina*) protein hydrolysates (MPh) were found to exert anti-diabetic and protective effects against liver and kidney damage in mice with type II diabetes adapted to a 45% kcal high-fat diet (HFD). The hypoglycemic, hepatoprotective and nephroprotective effects of MPh were analyzed after 12 weeks of the continuous oral administration of MPh at 125, 250 and 500 mg/kg. Diabetic control mice exhibited an increase in body weight, and blood glucose and insulin levels, with a decrease in serum high-density lipoprotein (HDL) levels. In addition, an increase in the regions of steatohepatitis, hepatocyte hypertrophy, and lipid droplet deposit-related renal tubular vacuolation degenerative lesions were detected, with noticeable expansion and hyperplasia of the pancreatic islets, and an increase in glucagon- and insulin-producing cells, insulin/glucagon cell ratios in the endocrine pancreas and hepatic lipid peroxidation, as well as decreased zymogen contents. Furthermore, a deterioration of the endogenous antioxidant defense system was observed, with reduced glucose utilization related hepatic glucokinase (GK) activity and an increase in hepatic glucoseogenesis-related phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6pase) activity. However, all of these diabetic complications were significantly inhibited by oral treatment with MPh in a dose-dependent manner. In addition, the marked dose-dependent inhibition of hepatic lipid peroxidation, the depletion of the liver endogenous antioxidant defense system, and changes in hepatic glucose-regulating enzyme activities were also observed. The results of this study suggest that MPh exerts potent anti-diabetic effects, along with the amelioration of related complications in mice with type II diabetes. The overall effects of MPh at a dose of 125 mg/kg on HFD-induced diabetes and related complications were similar or more potent than those of metformin (250 mg/kg).

Introduction

Recently, there has been a worldwide increase in the incidence of type II diabetes associated with obesity, the development of which seems to be due to a combination of high-caloric diets and physical inactivity (1,2). The predicted estimates suggest that the worldwide population with this syndrome may double to over 300 million by the year 2025 (3). Triglyceride (TG) accumulation in tissues, particularly in fatty tissues, is associated with the excessive intake of fatty acids. The excess of fatty acids in non-adipose tissues has been linked to the higher circulation of fatty acids and insulin (2). Insulin resistance has been shown to be associated with higher levels of fatty acid binding, and a higher availability of transport proteins in adipose and non-adipose tissues, which facilitate the uptake of fatty acid by these tissues. The higher levels of free fatty acid (FFA) deposition in muscle trigger a negative feedback in glucose utilization and the insulin-mediated muscle insulin signaling pathway (4). Prolonged exposure to FFAs in the pancreas may cause impaired insulin release through lipotoxicity (5). High FFA concentrations in the liver resist the action of insulin by enhancing glucose output (6). The accumulation of TGs in the liver causes non-alcoholic fatty liver disease (NAFLD). The liver is the main organ for glucose metabolism and NAFLD damages the liver via hepatocellular necrosis, steatosis, fibrosis and steatohepatitis (7). The imbalanced lipolysis and hepatic lipogenesis causes NAFLD and insulin resistance (8).

It has been reported that oxidative stress inhibition and the control of post-prandial hyperglycemia play an important...
role in the treatment of diabetes (9). Hence, safe and effective α-glucosidase inhibitors, antioxidants and functional food materials from natural sources have been the focus of research (10,11). For example, metformin, an orally administered anti-diabetic drug from the biguanide class has been in the treatment of type II diabetes (12). The most severe potential side-effect of metformin is lactic acidosis, although the occurrence of this phenomenon is very rare and it has been mostly related to comorbid conditions, i.e., impaired kidney or liver function (13).

Protein hydrolysates have evolved to the point where they are promising nutrient supplements for use in clinical and elemental nutrition, in the treatment of weight loss, as well as malnutrition associated with various clinical conditions (14). Fish hydrolysates, which have been suggested to be advantageous protein nutritional supplements for different medical conditions (15,16), also have antioxidant, antimicrobial and antiproliferative effects (17). In particular, some protein hydrolysates have been shown to exert hypolipidemic and anti-obesity effects in high-fat diet (HFD)-fed obese animal models (18-21). The melanian snail, *Semisulcospira libertina*, is one of the most commonly encountered freshwater snails in Far East Asia, and has been used as an ingredient of tonic functional foods in Korea (22-24).

The present study was performed to examine the pharmacological anti-diabetic activity, and ameliorating effects with respect to related complications, of melanian snail (*Semisulcospira libertina*) protein hydrolysates (MPh) in mice with type II diabetes.

**Materials and methods**

**Animals.** In total, 50 female specific pathogen-free (SPF) ICR mice (6 weeks old upon receipt; OrientBio, Seungnam, Korea) were used in the experiments after a 7-day period of acclimatization to the laboratory environment. The animals were allocated 4-5 per polycarbonate cage in a temperature (20-25°C)- and humidity (40-45%)-controlled room under a 12:12-h light:dark cycle with free access to commercial rodent chow (Samyang, Seoul, Korea) and water. The animals were allocated to 6 groups following a 1-week adaptation period (n=8 mice/group; total of 40 HFD-fed mice and 8 normal diet-fed mice as the intact group) based on body weight (intact control: mean, 29.60±1.59 g; HFD group: mean, 35.46±2.84 g). The formulas of the normal and high-fat diets used in this study are shown in Table I. All laboratory animals were treated according to the national regulations for the usage and welfare of laboratory animals, and the protocol was approved by the Institutional Animal Care and Use Committee of Daegu Haany University (encoded as MPh2015Ku01). Metformin hydrochloride (Wako, Osaka, Japan) was used as a reference.

MPh dissolved in distilled water was administered orally, at doses of 125, 250 and 500 mg/kg, once a day for 84 days after 7 days of HFD adaptation. Metformin dissolved in distilled water was also administered orally at 250 mg/kg. The intact vehicle and diabetic control mice received an oral administration of equal volumes of distilled water.

**Changes in body and organ weight.** Body weight was measured just before commencement of the HFD regime. One day before the initiation of MPh administration, on the first administration day (day 0), and then weekly until termination on day 84 using an automatic electronic balance (Precisa Instruments, Dietikon, Switzerland). At initiation and termination of administration, all experimental animals were fasted overnight with access only to water (approximately 12 h) to reduce differences due to feeding. In addition, body weight gain was calculated during the adaptation period (from day -8 to 0 of test agent administration) and administration period (from day 0-84 of test agent administration) as follows:

\[
\text{body weight gain (g): during the 7-day adaptation period} = \text{body weight at initiation of administration} - \text{body weight at initiation of HFD supply (from day -8 to 0 of test agent administration)}
\]

Table I. Formulas of normal and high-fat diets used in this study.

| Composition | Normal pellet diet (g/kg diet) | High-fat diet (g/kg diet)* |
|-------------|-------------------------------|---------------------------|
| Ingredient  |                               |                           |
| Casein      | 200                           | 200                       |
| L-cysteine  | 3                             | 3                         |
| Corn starch | 150                           | 72.8                      |
| Sucrose     | 500                           | 172.8                     |
| Cellulose   | 50                            | 50                        |
| Soybean oil | 50                            | 25                        |
| Lard        | 0                             | 177.5                     |
| Mineral mixture | 35                         | 10                        |
| Vitamin mixture | 10                          | 10                        |
| Choline bitartrate | 2                           | 2                         |
| Energy (kcal/g)       | 0.21                        | 4.73                      |
| Protein (% kcal/kg)   | 13.3                        | 20                        |
| Carbohydrate (% kcal/kg) | 47.4                      | 35                        |
| Fat (% kcal/kg)       | 8.0                         | 45                        |
| Fiber (% kcal/kg)     | 8.0                         | 8.0                       |

*Pellet diets 45% kcal/fat (D12451; Research Diet, New Brunswick, NJ, USA) were used as high-fat diet (HFD) and normal rodents pellet diet (Superfeed Co., Seoul, Korea) were used as normal fat pellet diets.
were determined by measuring the absorbance at 412 nm using 25% trichloroacetic acid (Merck, San Francisco, CA, USA), and centrifuged at 1,700 x g for 40 min at 4˚C. GSH contents were determined individually and to minimize the effect of inter-individual difference in body weight, the relative weights (% of body weight) were also calculated by means of body weight at sacrifice.

Mean daily food consumption measurements. Diets of 150 g in each individual cage were supplied, with the remaining amount of food measured after 24 h via an automatic electronic balance (Precisa Instruments). That amount was then divided by the number of animals reared in the cage. These measurements were conducted once a week for 84 days during the administration period, as previously described (25,26).

Measurement of serum biochemistry levels. For the measurement of blood glucose levels, blood samples were collected from the caudal vena cava into NaF glucose vacuum tubes (Becton-Dickinson, Franklin Lakes, NJ, USA) and plasma was separated at sacrifice. Blood glucose levels were measured using an automated blood glucose analyzer (200 FR; Toshiba, Tokyo, Japan). For other serum biochemistry measurements, blood samples were collected from the caudal vena cava into clotted activating serum tubes. The samples were centrifuged at 16,200 x g for 10 min at ambient temperature. The alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), blood urine nitrogen (BUN), TG, total cholesterol (TC), creatinine, low-density lipoprotein (LDL), high-density lipoprotein (HDL) cholesterol and cholesterol levels were measured using an automated blood analyzer (Hemagen Analyst Hemagen Diagnostic, Columbia, MD, USA), while serum LDL and HDL levels were detected using different blood analyzer (AU400; Olympus, Tokyo, Japan). Serum insulin and blood HbA1c levels were measured using a HbA1c measuring system (Infopia, Anyang, Tokyo, Japan). Serum LDL and HDL levels were measured using an automated blood system (Randox, Crumlin, UK), while serum insulin levels were measured using different blood analyzer (AU400; Olympus, Tokyo, Japan). Serum insulin and blood HbA1c levels were measured using a HbA1c measuring system (Infopia, Anyang, Tokyo, Japan).

Lipid peroxidation and liver antioxidant defense system. Before measuring the lipid peroxidation and antioxidant activities, organ weights were measured. Glutathione (GSH) and malondialdehyde (MDA) contents, and the enzyme activities of superoxide dismutase (SOD) and catalase (CAT) in mouse hepatic tissues, were measured. Liver tissues were weighed and homogenized in ice-cold 0.01 M Tris-HCl (pH 7.4), and centrifuged at 12,000 x g for 15 min, as previously described by Kavutcu et al (29). Lipid peroxidation in the liver was determined by estimating MDA contents using the thiobarbituric acid test at absorbance 525 nm as nM of MDA mg⁻¹ tissue (30). Total protein contents were measured using a previously described method (31) and bovine serum albumin (BSA) (Invitrogen, Carlsbad, CA, USA) was used as an internal standard. Prepared homogenates were mixed with 0.1 ml of 25% trichloroacetic acid (Merck, San Francisco, CA, USA), and centrifuged at 1,700 x g for 40 min at 4˚C. GSH contents were determined by measuring the absorbance at 412 nm using 2-nitrobenzoic acid (Sigma-Aldrich, St. Louis, MO, USA) as µM/mg tissue (32). H₂O₂ decomposition in the presence of CAT was measured at 240 nm, as previously described (33). CAT activity was measured as the amount of enzyme required to decompose 1 nM H₂O₂/min at 25˚C, pH 7.8 and the results are expressed as U/mg tissue. SOD activity was measured according to the method described by Sun et al (34). The estimation of SOD activity was based on superoxide radical generation by xanthine and xanthine oxidase, which react with nitrotriazolium blue to form formazan dye. SOD activity was measured at absorbance 560 nm as the degree of inhibition of this reaction, and was expressed as U/mg tissue. One unit of SOD enzymatic activity was equivalent to the amount of enzyme that reduced the initial absorbance of nitroblue tetrazolium by 50% in 1 min.

Measurement of hepatic glucose-regulating enzyme activities. The hepatic enzyme source was prepared based on the method described by Hulcher and Oleson (35). Briefly, 0.3 g of hepatic tissue was homogenized in buffer solution (0.1 M triethanolamine, 0.2 M EDTA and 0.002 M dithiothreitol), and centrifuged at 1,000 x g for 15 min at 4˚C. The supernatant was centrifuged again at 10,000 x g for 15 min at 4˚C. Glucokinase (GK) activity was measured according to the method described by Davidson and Arion (36) with slight modifications. Briefly, 0.98 ml of the reaction mixture [100 mM KCl, 50 mM NAD⁺, 50 mM HEPES-NaGT, pH 7.4, 10 mM glucose, 7.5 mM MgCl₂, 2.5 mM dithioerythritol, 10 µl of hepatic tissue homogenate, 4 units of glucose-6-phosphate dehydrogenase (G6PDH) and 10 mg/ml albumin], was pre-incubated at 37˚C for 10 min. The reaction was initiated by the addition of 10 µl of 5 mM ATP, and the mixture was incubated at 37˚C for 10 min. The variation in absorbance at 340 nm was recorded. Glucose-6-phosphate (G6pase) activity was measured by following the method described by Alegre et al (37). The reaction mixture contained 765 µl of 131.58 mM HEPES-NaGT (pH 6.5), 100 µl of 265 mM glucose-6-phosphate, 100 µl of 18 mM EDTA (pH 6.5), 10 µl of 0.2 M NADP⁺, 0.6 U/ml glucose dehydrogenase and 0.6 U/ml mutarotase. A total of 5 µl of the pre-incubated hepatic tissue homogenate at 37˚C was added to the mixture and incubated again at 37˚C for 4 min. The change in absorbance at 340 nm was recorded. The phosphoenolpyruvate carboxykinase (PEPCK) activity was assessed using the method described by Bentle and Lardy (38) method. The reaction mixture contained 500 mM NaHCO₃, 200 mM PEP, 100 mM IDP, 72.92 mM sodium HEPES (pH 7.0), 25 mM NADH, 10 mM MnCl₂, 10 mM dithiothreitol, 10 µl of hepatic tissue homogenate and 7.2 units of malic dehydrogenase. The hepatic enzyme activity was measured based on the decrease in absorbance of the mixture at 340 nm at 25˚C. All reagents and chemicals used in this hepatic enzyme activity measurement were obtained from Sigma-Aldrich.

Histopathology. After measuring organ weights, the splenic lobes of the pancreas and the left lateral lobes of the left kidney and liver were fixed in 10% neutral-buffered formalin, embedded in paraffin and cut into serial sections of 3-4 µm thickness. Representative sections were stained with hematoxylin and eosin (H&E, Sigma-Aldrich) for light microscopy (microscope: Eclipse 80i; Nikon Corp., Tokyo, Japan). The histological profiles of individual organs were then examined. Alternatively, the liver portions dehydrated in 30% sucrose solution were cut into frozen sections on a cryostat for staining...
with Oil Red O (Alfa Aesar, Heysham, UK), as previously described (39,40). To observe more detail histopathological changes, the steatohepatitic regions and mean hepatocyte diameter (H&E staining) were calculated by automated image analysis (iSolution FL ver. 9.1; IMT i-solution Inc., Vancouver, QC, Canada) on the restricted view fields according to previously described methods (25,26,39,40). Steatohepatitic regions, i.e., regions with fatty deposits in the hepatic parenchyma, were calculated as a percentage of lipid deposition regions on cryostat sections with Oil Red staining using a microscope with an automated image analysis program (% mm^2 of hepatic parenchyma). The mean diameter (µm) of hepatocytes was also calculated by an automated image analysis program using H&E staining; at least 10 hepatocytes were examined per view field of the liver. In addition, means of vacuolated renal tubules with lipid droplet deposition were calculated using an automated image analysis program (number 100^2 tubules; one field/sample); the mean area occupied by zymogen granules (% mm^2 of pancreatic parenchyma), diameters (µm) of pancreatic islets, as well as the numbers (islets/10 mm^2 of pancreatic parenchyma) were also measured using an automated image analysis program according to previously established methods (25,26,40). The histopathologist was blinded to group allocation at the time of the analysis.

**Immunohistochemistry.** Serial sections of other pancreatic tissues (remaining tissue after H&E staining) were immunostained by the avidin-biotin-peroxidase (ABC) method (40) using rabbit polyclonal anti-glucagon (dilution 1:2,000; Cat. no. ab133195, Abcam, Cambridge, MA, USA) or guinea pig polyclonal anti-insulin (dilution 1:2000; Cat. no. ab7842, Abcam) (both from DiaSorin, Stillwater MN, USA) antiserum. Briefly, endogenous peroxidase activity was blocked by incubating in 0.3% H2O2 and methanol for 30 min, and non-specific binding of immunoglobulin was blocked with normal horse serum blocking solution (dilution 1:100; Vector Laboratories, Burlingame CA, USA) for 1 h in a humid chamber. Treatment with primary antiserum was performed overnight at 4°C in a humid chamber, followed by incubation with biotinylated universal secondary antibody (dilution 1:50; Cat. no. PK-6200; Vector Laboratories) and ABC reagents (dilution 1:50, Vectastain Elite ABC kit; Vector Laboratories) for 1 h at room temperature in a humid chamber. Finally, the sections were reacted with the peroxidase substrate kit (Vector Laboratories) for 3 min at room temperature. All sections were rinsed 3 times in 0.01 M phosphate-buffered saline (PBS) between steps. Immunoreactive cell densities over 20% as compared with other naïve cells were regarded as positive, and the positive cells were assessed based on mean areas of pancreatic islets (mm^2) using the automated image analysis, as previously described (41,40). The ratios of insulin-positive/glucagon-positive cells were calculated as shown below in equation [2]. The histopathologist was blinded to the group allocation at the time of the analysis.

**Equation [2]:**

\[
\text{Insulin positive/glucagon positive cells (ratio)} = \frac{A}{B}
\]

where, A represents the mean number of insulin immunoreactive cells, and B represents the mean number of glucagon immunoreactive cells.

**Statistical analyses.** All numerical values are expressed as the means ± standard deviation (SD) of 8 mice. Multiple comparison tests of the different dose groups were conducted. Homogeneity of variance was examined using Levene's test (42). In case of no significant deviations detected from homogeneity of variance by Levene's test, the data were analyzed by one-way ANOVA followed by the least-significant differences multiple-comparison (LSD) test to determine the significantly different group pairs. In case of significant deviations from homogeneity of variance on Levene's test, the non-parametric Kruskal-Wallis H test was conducted. When a significant difference was observed on the Kruskal-Wallis H test, the Mann-Whitney U test was performed to determine the significantly different specific group pairs. Statistical analyses were performed using SPSS software (ver. 14.0; SPSS Inc., Chicago, IL, USA), as previously described (43). The efficacy of the test agents was calculated by comparing the percentage changes with HFD control, and the assess disease induction was assessed by calculating the percentage changes between intact and HFD controls according to equations [3] and [4], as previously described (40).

**Equation [3]:**

\[
\% \text{ changes compared with intact control} = \frac{\text{Data of HFD control} - \text{Data of intact control}}{\text{Data of intact control}} \times 100
\]

**Equation [4]:**

\[
\% \text{ changes compared with HFD control} = \frac{\text{Data of test agent treated mice} - \text{Data of HFD control}}{\text{Data of HFD control}} \times 100
\]

**Results**

**Changes in body weight and food consumption.** We selected only adapted mice exhibiting a regular body weight increase as compared to the intact (normal diet) controls during 1 week of HFD supply (intact control: mean, 29.60±1.59 g; range, 27.30-32.20 g; HFD group: mean, 32.33±1.63 g; range, 29.70-35.80 g). HFD control mice exhibited significant (p<0.01) increases in body weight compared with the intact controls from 1 week after commencement of being fed the HFD; accordingly, body weight gains during the 7-day HFD adaptation period and 84-day administration period were also significantly increased compared with the intact controls (both p<0.01). However, significant (p<0.01 or p<0.05) decreases in body weight were detected in mice treated with metformin at 250 mg/kg, or MPh at 125, 250 and 500 mg/kg; accordingly, body weight gains during the 84 days of administration were also significantly decreased in these groups compared with the HFD controls (all p<0.01) (Table II).

Although a significant (p<0.01) decrease in mean daily food consumption was detected in all HFD-fed mice compared with the intact controls, no significant changes were observed in mean daily food consumption in any of the test agent groups (Table II).

**Anti-diabetic effects**

**Effects on pancreatic weight changes.** A significant (p<0.01) decrease in the relative pancreas weight was identified in the HFD control mice compared with the intact controls. However,
Metformin 1.334±0.134
Reference levels were significantly (p<0.01) decreased by treatment with metformin or MPh compared with the intact controls. However, the serum insulin in serum insulin levels was detected in the HFD controls decreased by treatment with metformin or MPh compared with the intact controls. However, the blood glucose levels were significantly (p<0.01) noted in the HFD controls compared with the intact controls.
Significant (p<0.01) increase in blood glucose levels was noted in the HFD controls, compared with the intact controls (Table III).

HFD controls (Table IV and Fig. 2).

Significantly (p<0.01 or p<0.05) reduced by treatment with all test agents, including MPh at 125 mg/kg, MPh at 250 mg/kg or MPh 500 mg/kg, compared with the HFD controls. No significant changes were detected in the absolute pancreatic weight in any of the experimental HFD-fed mice, including HFD controls, compared with the intact controls (Table III).

Effects on blood glucose, insulin and HbA1c levels. A significant (p<0.01) increase in blood glucose levels was noted in the HFD controls compared with the intact controls. However, the blood glucose levels were significantly (p<0.01) decreased by treatment with metformin or MPh compared with the HFD controls (Fig. 1A). A significant (p<0.01) increase in serum insulin levels was detected in the HFD controls compared with the intact controls. However, the serum insulin levels were significantly (p<0.01) decreased by treatment with all test agents compared with the HFD controls (Fig. 1B). In addition, a significant (p<0.01) increase in the blood HbA1c content was observed in the HFD controls compared with the intact controls. However, the blood HbA1c content was significantly (p<0.01) decreased by treatment with all test agents compared with the HFD controls (Fig. 1C).

Effects on pancreatic islet hyperplasia and expansion. A significant (p<0.01) increase in the number and mean diameter of pancreatic islets was detected in the HFD controls compared with the intact controls due to marked hyperplasia of the pancreatic islets themselves or component endocrine cells. However, both hyperplasia and expansion of islets were significantly (p<0.01 or p<0.05) reduced by treatment with all test agents, including MPh at 125 mg/kg, compared with the HFD controls (Table IV and Fig. 2).

Table II. Changes on body weight gain and mean daily food consumption in mice with type II diabetes.

| Group      | Body weight gain during | Body weight (g) at days after initial test substance treatment |
|------------|-------------------------|---------------------------------------------------------------|
|            | Adaptation period [B-A] | Mean daily food consumption (g) |
|            | Administration period [D-C] | 8 days | 1 day | 0 day$^a$ | 84 days$^b$ |
| Controls   |                         |                                             |                                             |             |
| Intact     |                         |                                             |                                             |             |
| HFD        |                         |                                             |                                             |             |
| Reference  |                         |                                             |                                             |             |
| Metformin  |                         |                                             |                                             |             |
| MPh-treated|                         |                                             |                                             |             |

$^a$All animals were fasted overnight; $^p$<0.05 as compared with the intact control by LSD test; $^p$<0.01 as compared with HFD control by MW test. Values are expressed as the means ± SD of 8 mice. HFD, 45% kcal high-fat diet; MPh, melanian snail (Semisulcospira libertina) protein hydrolysates, test material. Metformin was administered at dose levels of 250 mg/kg.

Table III. Changes on absolute and relative organ weights in mice with type II diabetes.

| Group      | Absolute organ weight (g) | Relative organ weight (% of body weight) |
|------------|---------------------------|-----------------------------------------|
|            | Liver                     | Kidney                                  | Pancreas                                |
|            | Liver                     | Kidney                                  | Pancreas                                |
| Controls   |                           |                                         |                                         |
| Intact     | 1.174±0.063               | 0.177±0.008                             | 0.201±0.023                             |
| HFD        | 1.729±0.109               | 0.245±0.015                             | 0.187±0.019                             |
| Reference  |                           |                                         |                                         |
| Metformin  | 1.334±0.134$^{a,e}$       | 0.206±0.026$^{a,g}$                     | 0.188±0.009                             |
| MPh-treated|                           |                                         |                                         |
| 500 mg/kg  | 1.140±0.110$^{c}$         | 0.180±0.008$^{f}$                      | 0.197±0.019                             |
| 250 mg/kg  | 1.180±0.008$^{c}$         | 0.186±0.008$^{e,f}$                    | 0.192±0.020                             |
| 125 mg/kg  | 1.307±0.086$^{b,c}$       | 0.201±0.010$^{d,f}$                     | 0.188±0.010                             |

$^a$<0.01 and $^p$<0.05 as compared with the intact control by LSD test; $^p$<0.01 as compared with HFD control by LSD test; $^p$<0.01 and $^p$<0.05 as compared with the HFD control by Mann-Whitney U (MW) test; $^p$<0.01 and $^p$<0.05 as compared with the HFD control by MW test. Values are expressed as the mean ± SD of 8 mice. HFD, 45% kcal high-fat diet; MPh, melanian snail (Semisulcospira libertina) protein hydrolysates, test material. Metformin was administered at dose levels of 250 mg/kg.

A significant (p<0.01) increase in relative pancreas weight was noted in the mice treated with metformin at 250 mg/kg or MPh at 125, 250 and 500 mg/kg compared with the HFD controls. No significant changes were detected in the absolute pancreatic weight in any of the experimental HFD-fed mice, including HFD controls, compared with the intact controls (Table III).
Effects on pancreatic islet glucagon- and insulin-immunoreactive cells. A significant (p<0.01) increase in glucagon- and insulin-immunoreactive cells, and in the insulin/glucagon-immunoreactive cell ratio, was noted in the HFD control mice compared with the intact controls. However, these abnormal increases in insulin- and glucagon-immunoreactive cells and their ratios (insulin-immunoreactive/glucagon-immunoreactive cells) were significantly (p<0.01) normalized by treatment with all test agents, including MPh at 250 mg/kg, compared with the HFD controls (Table IV and Fig. 3).

Antihyperlipidemic effects
Effects on serum TC, TG, LDL, and HDL levels. A significant (p<0.01) increase in serum TC, TG and LDL levels was detected in the HFD controls compared with the intact controls. However, the serum HDL levels were significantly (p<0.01) increased in all of the test agent groups (Table V). Metformin was administered at dose levels of 250 mg/kg.

Effects on glucose-regulating enzymes in the liver
Effects on hepatic GK activity. A significant (p<0.01) decrease in the activity of hepatic GK, one of the blood glucose-utilizing hepatic enzymes, was noted in the HFD controls compared with the intact controls; however, these levels were significantly (p<0.01) normalized by treatment with all test agents compared with the HFD control mice (Table VI).
Effects on hepatic G6pase activity. A significant (p<0.01) increase in the activity of hepatic G6pase, one of the gluconeogenesis hepatic enzymes, was identified in the HFD controls compared with the intact controls; however, these levels were significantly (p<0.01) normalized by treatment with all test agents compared with the HFD control (Table VI).

Effects on liver weight. A significant (p<0.01) increase in absolute liver weight was observed in the HFD controls compared with the intact controls. However, this increase in absolute liver weight was significantly (p<0.01) normalized by treatment with...
all test agents, including MPh at 250 mg/kg, compared with the HFD control mice. No significant changes in relative liver weight were observed in the HFD control mice compared with the intact controls, and mice treated with any of the test agents compared with the HFD controls (Table III).

**Effects on serum AST and ALT levels.** A significant (p<0.01) increase in serum AST and ALT levels was observed in the HFD control mice as compared to the intact controls. However, the serum AST and ALT levels were significantly (p<0.01) decreased in all the test agent-administered mice (Table VI).

**Effects on steatohepatitis and hepatocyte hypertrophy.** A significant (p<0.01) increase in steatohepatitis (percentage change of fatty regions in liver parenchyma) was noted in the HFD controls as compared to the intact controls. This
Table V. Changes in the levels of secondary liver damage markers in mice with type II diabetes.

| Group      | AST (IU/l) | ALT (IU/l) | TC (mg/dl) | TG (mg/dl) | LDL cholesterol (mg/dl) | HDL cholesterol (mg/dl) | Liver steatosis (%/mm² of hepatic tissues) | Mean hepatocyte diameters (µm/cell) |
|------------|------------|------------|------------|------------|-------------------------|-------------------------|------------------------------------------|----------------------------------|
| Controls   |            |            |            |            |                         |                         |                                          |                                  |
| Intact     | 75.88±14.36| 36.25±12.01| 118.50±22.55| 52.63±18.32| 15.25±2.38              | 107.00±24.93            | 7.28±2.66                                 | 17.91±1.90                       |
| HFD        | 230.63±28.91| 176.13±21.12| 289.75±30.06| 218.13±18.73| 51.00±8.19              | 21.00±7.89              | 78.95±11.01                              | 55.14±12.63                       |

Reference

Metformin 152.00±45.23<sup>a</sup> 99.63±19.67<sup>c</sup> 207.38±39.19<sup>c</sup> 147.63±15.65<sup>c</sup> 28.88±8.63<sup>c</sup> 58.88±18.88<sup>c</sup> 42.82±14.23<sup>c</sup> 28.71±1.70<sup>c</sup>

MPh-treated 500 mg/kg 114.38±38.44<sup>bc</sup> 61.63±15.13<sup>bc</sup> 115.18±17.02<sup>bc</sup> 104.63±20.83<sup>bc</sup> 18.88±2.10<sup>bc</sup> 81.13±12.23<sup>bc</sup> 15.07±3.91<sup>bc</sup> 20.88±4.92<sup>bc</sup>

250 mg/kg 121.75±37.92<sup>bc</sup> 72.75±17.65<sup>bc</sup> 171.13±20.19<sup>bc</sup> 114.63±27.33<sup>bc</sup> 20.50±2.33<sup>bc</sup> 76.50±15.43<sup>bc</sup> 19.02±3.53<sup>bc</sup> 21.04±2.52<sup>bc</sup>

125 mg/kg 146.13±38.36<sup>bc</sup> 90.13±17.88<sup>bc</sup> 192.13±29.35<sup>bc</sup> 139.00±19.79<sup>bc</sup> 27.50±5.48<sup>bc</sup> 68.75±14.85<sup>bc</sup> 37.79±12.52<sup>bc</sup> 26.13±6.18<sup>bc</sup>

*p<0.01 and <sup>b</sup>p<0.05 as compared with the intact control by LSD test; <sup>c</sup>p<0.01 as compared with the HFD control by LSD test. Values are expressed as the means ± SD of 8 mice. HFD, 45% kcal high-fat diet; MPh, melanian snail (*Semisulcospira libertina*) protein hydrolysates, test material. Metformin was administered at dose levels of 250 mg/kg; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TC, total cholesterol; TG, triglyceride; LDL, low density lipoprotein; HDL, high density lipoprotein.

Table VI. Changes in hepatic glucose-regulating enzyme activities in mice with type II diabetes.

| Group      | Glucokinase (nM/min/mg protein) | Glucose-6-phosphatase (nM/min/mg protein) | PEPCK (nM/min/mg protein) |
|------------|---------------------------------|------------------------------------------|--------------------------|
| Controls   |                                 |                                          |                          |
| Intact     | 2.93±0.28                       | 106.12±16.57                            | 1.84±0.40                |
| HFD        | 1.71±0.37<sup>a</sup>           | 194.17±23.69                            | 4.91±0.79<sup>a</sup>    |

Reference

Metformin 2.34±0.27<sup>ac</sup> 143.48±21.87<sup>ac</sup> 2.99±0.65<sup>ac</sup>

MPh-treated 500 mg/kg 2.85±0.32<sup>c</sup> 120.64±17.81<sup>ac</sup> 2.40±0.43<sup>c</sup>

250 mg/kg 2.65±0.46<sup>c</sup> 132.41±21.62<sup>ac</sup> 2.63±0.42<sup>c</sup>

125 mg/kg 2.39±0.42<sup>c</sup> 139.86±26.50<sup>ac</sup> 2.91±0.74<sup>c</sup>

*p<0.01 and <sup>c</sup>p<0.05 as compared with the intact control by LSD test; <sup>a</sup>p<0.01 as compared with the HFD control by LSD test. Values are expressed as the means ± SD of 8 mice. HFD, 45% kcal high-fat diet; MPh, melanian snail (*Semisulcospira libertina*) protein hydrolysates, test material; PEPCK, phosphoenolpyruvate carboxykinase. Metformin was administered at dose levels of 250 mg/kg.

was perhaps due to the severe hypertrophy of hepatocytes related to intracellular lipid depositions. However, increases in steatohepatitis were significantly (p<0.01) normalized by treatment with all test agents, including metformin at 250 mg/kg. A significant (p<0.01) increase in the mean diameter of hepatocytes (hypertrophy) was observed in the HFD controls compared with the intact controls. However, hepatocyte hypertrophy was markedly and significantly (p<0.01) decreased in all test agent-treated mice, including the MPh 125 mg/kg group, compared with the HFD controls (Table V and Fig. 4).

Effects on kidney damage
Effects on kidney weight. A significant (p<0.01) increase in absolute kidney weight was observed in the HFD controls compared with the intact controls; this increase was significantly (p<0.01 or p<0.05) normalized by treatment with all test agents, including MPh at 500 mg/kg, compared with the HFD-fed mice. No significant changes in relative kidney weight were noted in the HFD control mice compared with the intact controls, and there were no significant changes in relative kidney weight in mice treated with any of the test agents compared with the HFD control mice (Table III).

Effects on serum BUN and creatinine levels. A significant (p<0.01) increase in serum BUN levels was observed in the HFD controls compared with the intact controls. However, the serum BUN levels were significantly (p<0.01) decreased in the HFD-fed mice treated with all test agents compared with the HFD controls (Table VII). In addition, a significant (p<0.01) increase in serum creatinine levels was noted in the HFD control mice compared with intact the controls. However, the serum creatinine levels were significantly (p<0.01) decreased in all test agent-treated HFD mice, including the MPh group at 500 mg/kg group, when compared with the HFD controls (Table VII).

Effects on kidney histopathology. A significant (p<0.01) increase in degenerative vacuolated renal tubules was observed in the HFD control mice compared with the intact controls, which resulted from diabetic nephropathies associated with lipid droplet deposition; however, these diabetic nephropathies were significantly (p<0.01) normalized by treatment with all test agents in the experiments (Table VII and Fig. 5).

Effects on the antioxidant system of the liver
Effects on liver lipid peroxidation. A significant (p<0.01) increase in liver lipid peroxidation and the elevation of hepatic MDA content was noted in the HFD controls compared with the intact controls; however, these increases...
were significantly (p<0.01) normalized by treatment with all test agents, including MPh at 125 mg/kg, compared with the HFD controls (Table VIII).

Effects on the GSH contents and on hepatic CAT and SOD activities. A significant (p<0.01) decrease in the hepatic GSH content, and CAT and SOD enzymatic activity, as representative endogenous antioxidants, was observed in the HFD control mice compared with the intact controls. However, the hepatic GSH content was significantly (p<0.01) increased in all test agent-treated HFD mice (Table VIII). In addition, the decreases in hepatic CAT and SOD activities were significantly (p<0.01) normalized by treatment with all test agents (Table III).
examine the pharmacological activities of MPh in mice fed a HFD, leading to mild diabetes and obesity (25,28,45,46). Metformin, a representative anti-diabetic drug used in the treatment of type II diabetes (47,48), was used as a potent reference agent.

HFD-fed animals exhibit mild obesity and hyperglycemia; therefore, models using these animals are appropriate for the development of preventive agents for metabolic syndromes (48). In the present study, the HFD control mice exhibited a significant increase in body weight as compared to the intact mice 1 week after the commencement of HFD feeding; accordingly, body weight gains during the 7-day HFD adaptation period and 84-day administration period were also significantly increased compared with the intact controls. However, these increases in body weight were significantly and dose-dependently inhibited by treatment with MPh at 125, 250 and 500 mg/kg, and also by metformin at 250 mg/kg.

The energy content of the HFD (4.73 kcal/kg) was much higher (approximately 20-fold) (Table I) than that of the normal diet (0.21 kcal/g); hence, the decrease in mean daily food consumption observed in all the diabetic mice compared with the intact controls was considered not to be a critical issue. A similar decrease has previously been reported in the daily food consumption of HFD-fed mice (26,40). In the present study, no significant changes in the levels of mean daily food consumption were detected in any of the test agent-administered groups compared with the HFD controls, suggesting that the pharmacological effects of the test agents detected in this study were unlikely to have been due to the inhibition of food consumption.

Marked increases in blood glucose, insulin and HbA1c levels, together with increases in insulin- and glucagon-immunoreactive cells, pancreatic islet numbers and diameters, and the insulin/glucagon cell ratio upon histopathological observation, were detected in the HFD control mice compared with the intact controls, similar to the insulin resistance observed in type II diabetes. However, all three doses of MPh effectively and dose-dependently inhibited these increases in insulin,

Table VII. Changes in the levels of secondary kidney damage markers in mice with type II diabetes.

| Group          | BUN (mg/dl) | Creatinine (mg/dl) | Degenerative renal tubule numbers (%) |
|----------------|-------------|--------------------|---------------------------------------|
| Controls       |             |                    |                                       |
| Intact         | 37.00±13.05 | 0.69±0.20          | 3.88±1.89                             |
| HFD            | 103.00±21.67| 2.25±0.32          | 75.75±14.19                          |
| Reference      |             |                    |                                       |
| Metformin      | 68.25±15.94 | 1.56±0.32          | 44.63±15.19                          |
| MPh-treated    |             |                    |                                       |
| 500 mg/kg      | 41.75±10.79 | 0.94±0.12          | 21.00±3.93                           |
| 250 mg/kg      | 57.75±14.24 | 1.03±0.18          | 30.13±6.13                           |
| 125 mg/kg      | 66.13±11.34 | 1.43±0.33          | 41.75±9.32                           |

Values are expressed as the means ± SD of 8 mice. HFD, 45% kcal high-fat diet; MPh, melanian snail (Semisulcospira libertina) protein hydrolysates, test material. Metformin was administered at dose levels of 250 mg/kg. *p<0.01 and **p<0.05 as compared with the intact control by Mann-Whitney U (MW) test; ***p<0.01 as compared with the HFD control by MW test.

Discussion

Diabetes mellitus is a major human health concern due to its increasing prevalence, debilitating complications and chronic course (44). The inhibition of oxidative stress and postprandial hyperglycemia are considered important for the treatment of diabetes (40,44). There has been a great deal of research aiming to identify safe and effective α-glucosidase inhibitors and antioxidants from natural materials to develop compounds or physiological functional foods for the treatment of diabetes (10,11,25,26,40). The present study was performed to examine the pharmacological activities of MPh in mice fed

Table VIII. Changes in liver lipid peroxidation and antioxidant defense systems in mice with type II diabetes.

| Group          | Lipid peroxidation | Antioxidant defense system |
|----------------|--------------------|---------------------------|
|                | Malondialdehyde (nM/mg tissue) | Glutathione (µM/mg tissue) | Catalase (U/mg tissue) | SOD (U/mg tissue) |
| Controls       |                    |                          |                          |                  |
| Intact         | 11.93±1.66         | 37.49±8.75              | 32.58±2.74              | 3.47±0.85        |
| HFD            | 32.31±6.27         | 10.38±2.19              | 9.94±2.65               | 0.82±0.09        |
| Reference      |                    |                          |                          |                  |
| Metformin      | 24.58±5.28         | 18.44±2.95              | 16.90±1.61              | 1.71±0.52        |
| MPh-treated    |                    |                          |                          |                  |
| 500 mg/kg      | 15.56±3.06         | 28.67±5.68              | 23.69±4.11              | 2.20±0.39        |
| 250 mg/kg      | 17.98±1.61         | 23.85±3.93              | 19.71±1.89              | 2.02±0.21        |
| 125 mg/kg      | 23.32±5.01         | 19.84±3.49              | 17.13±3.00              | 1.78±0.34        |

*p<0.01 as compared with the intact control by LSD test; **p<0.01 as compared with the HFD control by LSD test; ***p<0.01 and ****p<0.05 as compared with intact control by MW test; *****p<0.01 as compared with HFD control by Mann-Whitney U (MW) test. Values are expressed as the mean ± SD of 8 mice. HFD, 45% kcal high-fat diet; MPh, melanian snail (Semisulcospira libertina) protein hydrolysates, test material; SOD, superoxide dismutase. Metformin was administered at dose levels of 250 mg/kg.
HbA1c content and blood glucose levels, as well as abnormal endocrine histopathological changes in the pancreas (Table IV and Fig. 1). HbA1c is a form of hemoglobin that is measured primarily to identify the average plasma glucose concentration over prolonged periods, and is produced by erythrocytes with long-term exposure to high levels of glucose (49,50). It has been reported that the hyperglycemia, a main sign of diabetes, should be controlled to treat the disease (25,26,40,51). The HFD-fed mouse has been used as an animal model of type II diabetes and also exhibits noticeable hyperglycemia (25,26,45,46,52).
Long-term HFD feeding revealed the characteristics of type II diabetes, altered HbA1Ac levels and marked elevations in the blood. In addition, increased insulin secretion is related to pancreatic islet hyperplasia commensurate with the progression of insulin resistance caused by HFD feeding (53-55). In previous studies, insulin-producing cells and total pancreatic islet numbers were increased after chronic consumption of HFD, with the islets increasing in area and number to secrete more insulin for maintenance of glucose homeostasis (56), followed by noticeable hypertrophy or hyperplasia of endocrine pancreas cells (53-56). These findings directly indicate that MPh exerts obvious hypoglycemic effects in mice, possibly through the inhibition of pancreatic endocrine changes.

Hyperlipidemia also generally occurs with the chronic progression of diabetes in HFD-fed mice (44). As the most critical issues in hyperlipidemia are the increases in serum TG, TC and LDL levels with decreased HDL levels (25,26,57) the efficacy of hypolipidemic agents is generally evaluated based on decreases in serum TC, TG and LDL along with increases in HDL levels (25,26,40,58). In the present study, all doses of MPh effectively and dose-dependently decreased the serum TC, LDL and TG levels, while increasing serum HDL levels compared with the HFD control mice, suggesting that MPh exerts favorable hypolipidemic effects on HFD-fed mice. In particular, in this study, MPh at 125 mg/kg exerted hypolipidemic effects comparable to those of metformin at 250 mg/kg on HFD-fed mice. In addition, it has been reported that HFD feeding results in the development of acinar cell atrophy, pancreatic steatosis and a decrease in the number of zymogen granules (40,59,60). Increases in the numbers of zymogen granules in exocrine pancreatic acinar cells indicate the production of digestive enzymes, particularly for digestion of proteins and lipids (61). In the present study, a reduction in the number of pancreatic zymogen granules was also detected histopathologically in the HFD-fed control group compared with the intact controls. However, these reductions in zymogen deposition in the exocrine pancreas were effectively inhibited, in a dose-dependent manner, by metformin at 250 mg/kg and also by treatment with MPh at 125, 250 and 500 mg/kg. These results may have direct evidence for the anti-hyperlipidemic effects of MPh in mice, which could be mediated by inhibition of lipid digestion caused by decrease in pancreatic enzyme release or production. As we could not completely exclude the possibility that MPh induced increases in digestive tract motility, further detailed studies are required to elucidate the precise mechanisms of action of MPh.

The hepatic enzyme, GK, is related to glucose homeostasis and its increased expression, which could cause an increase in blood glucose utilization for energy production or glycogen storage in the liver, leading to a reduction in blood glucose levels (62,63). By contrast, the enzymes, PEPCK and G6pase, are associated with hepatic glucose output and gluconeogenesis and increases in their activities are correlated with elevated glucose levels (64,65). Generally, noticeable decreases in hepatic GK activities, and increases in PEPCK and G6pase activities, are observed with HFD feeding (27), and were also observed in the HFD control mice in this study. All three doses of MPh effectively inhibited HFD-induced hepatic glucose-regulating enzyme activity changes in a dose-dependent manner. These results were considered direct evidence that MPh has favorable effects on the activities of hepatic glucose-regulating enzymes and, by extension, on the control of blood glucose levels.

With the progression of diabetes, increases in liver weight due to abnormal glycosylation-related hepatosteatosis, fibrosis and/or hepatocyte hypertrophic changes were observed, with elevation of serum ALT and AST levels (26,40,66). These phenomena have been regarded as diabetic hepatopathy, and were observed previously in HFD-fed mice (26,67). Improvements in these abnormal changes have been considered as direct evidence for the improvement of diabetic hepatopathy (66). In this study, all three doses of MPh effectively and dose-dependently decreased diabetic hepatopathy compared with HFD control mice, suggesting that they had favorable hepatoprotective effects. In addition, increases in kidney weight due to inflammation, necrotic processes and swelling were observed with the elevation of creatinine and serum BUN levels in chronic diabetes. The attenuation of these abnormal changes are considered as direct evidence of the amelioration of diabetic nephropathy (26,40). In this study, HFD-fed mice exhibited a marked increase in absolute kidney weight, elevated creatinine and serum BUN levels, and lipid droplet deposition-related renal tubule vacuolation upon histopathological observations. This suggests mild diabetic nephropathy; however, these levels were normalized by treatment with metformin and all three doses of MPh, representing direct evidence of the favorable nephroprotective effects of these agents.

There is considerable evidence of the roles of free radicals in altered antioxidant defenses in diabetes and the etiology of diabetes (68). Oxidative stress has been reported to play an important role in diabetes mellitus. The generation of free radicals by hyperglycemia is related to glucose autooxidation. Glucose autooxidation has been linked to non-enzymatic glycosylation, and glycosylated proteins have been reported to be a source of free radicals (reactive oxygen species (ROS)) (40,69). In addition, various toxic products of lipid peroxidation damage the surrounding tissues (70). Elevated lipid peroxidation, observed in various organs in HFD-fed mice, also acts as a potent redox cyclers by generating harmful ROS and causing organ damage (71,72). Oxidative stress in diabetes co-exists with a decrease in the antioxidant status (73), which can increase the deleterious effects of free radicals. The generation of ROS-related oxidative stress also plays an important role in the etiology of diabetic complications (74). Therefore, the endogenous antioxidant content, the degree of lipid peroxidation, GSH and activities of the antioxidant enzymes, CAT and SOD, in the liver tissue are of secondary importance to improve diabetes and various related complications (75,76). GSH is a representative endogenous antioxidant that prevents tissue damage by maintaining ROS at low levels (at certain cellular concentrations), and is accepted as a protective endogenous antioxidant factor in tissues (77). CAT is an enzyme that catalyzes the conversion of H2O2 to H2O and SOD is an antioxidant enzyme that contributes to enzymatic defense mechanisms (78). The depletion of GSH content, the marked elevation of hepatic lipid peroxidation and decreases in CAT and SOD activities were noted in HFD controls in the present study (25,79). However, MPh at 125, 250 and 500 mg/kg dose-dependently and effectively inhibited the deterioration of hepatic antioxidant defense systems compared with the HFD control mice, suggesting favorable antioxidant effects of MPh in mice.
In this study, MPh exerted potent anti-diabetic and ameliorating effects on mice with diabetic complications, through the increased modulation of antioxidant defense systems, hepatic glucose-regulating enzyme activities and pancreatic lipid digestion enzymes. The overall effects of MPh at 125 mg/kg on HFD-induced diabetes and related complications were similar or more potent than those of metformin at 250 mg/kg. Therefore, MPh is a promising, potent and novel medicinal food or ingredient for the treatment of type II diabetes and its related complications.

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