Antidiabetic Effects of Vigna nakashimae Extract in db/db Mice

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The inhibitory activity of Vigna nakashimae extract against intestinal α-glucosidase was investigated in vitro and in vivo. The extract exerted a significant inhibitory effect against intestinal α-glucosidases. With sucrose-loading, it reduced the peak responses of blood glucose significantly in normal mice. Next, it was administrated to 8-week-old db/db mice for 2 weeks, and then plasma glucose, triglyceride, and total cholesterol levels were measured. The extract significantly suppressed postprandial hyperglycemia and blood glycated hemoglobin in the db/db mice. In addition, it lowered fasting glucose and improved glucose tolerance. Furthermore, it led to significant decreases in plasma triglyceride levels. It reduced endoplasmic reticulum stress in thapsigargin-induced HepG2 cells. Taken together, these results suggest that Vigna nakashimae extract has hypoglycemic and hypolipidemic effects that occur via inhibition of α-glucosidase activity and endoplasmic reticulum stress.

Key words: type 2 diabetes; Vigna nakashimae; postprandial hyperglycemia; α-glucosidase inhibitory activity; endoplasmic reticulum stress

Type 2 diabetes is a multi-factorial, heterogeneous group of disorders characterized by a deficiency in or failure to maintain normal glucose homeostasis. For the most part, it results from defects in insulin secretion and insulin action. The prevalence of type 2 diabetes has increased sharply in recent decades, tracking similar increases in the prevalence of obesity, one of the primary risk factors. More than 171 million people worldwide are currently believed to be afflicted with type 2 diabetes, and it is estimated that this number will rise to about 366 million by 2030. Controlling hyperglycemia is the most important factor in reducing the risks associated with diabetes and diabetic complications. Both fasting and postprandial glucose are critical to achieving long-term proper control of hyperglycemia in diabetic patients. Although optimizing both fasting blood glucose and postprandial glucose levels is important for achieving near-normal glucose levels, it has been reported that postprandial glucose levels might be a better marker of glycemic control than fasting blood glucose levels.1–5) Additionally, several drugs have been developed to improve postprandial hyperglycemia by inhibiting intestinal α-glucosidase activity.

During the last few decades, α-glucosidase and aldose reductase inhibitors have received a great deal of attention due to their important roles in carbohydrate digestion and their therapeutic potential for the reduction of diabetic complications such as neuropathy, nephropathy, retinopathy, keratopathy, angiopathy, and cataracts.6) α-Glucosidase is located in the brush-border surface membrane of intestinal cells, and is the key enzyme involved in the catalysis of the final step of the digestion of carbohydrates. It specifically hydrolyzes the α-glucopyranosidic bond, releasing an α-D-glucose from the non-reducing end of the sugar. Hence α-glucosidase inhibitors can retard the liberation of D-glucose of oligosaccharides and disaccharides from dietary complex carbohydrates and delay glucose absorption, resulting in reduced postprandial plasma glucose levels and suppressed postprandial hyperglycemia.7,8) Many attempts have been made to identify effective and safe α-glucosidase inhibitors from natural sources to develop physiological functional foods or lead compounds for use against diabetes. Acarbose,9) voglibose,10) nojirimycin,11,12) and 1-deoxynojirimycin13) from natural sources are representative anti-diabetic agents that act on blood glucose levels after food intake.

Vigna species are an important source of protein for humans, particularly in tropical Africa and Asia, and several Vigna species have been domesticated in Asia. Among these, the cultigens, mungbean [V. radiata (L.) Wilczek], black gram [V. mungo (L.) Hepper] and azuki bean [V. angularis (Willd.) Ohwi and Ohashi] are the most important economically. In addition, the rice bean [V. umbellate (Thunb.) Ohwi and Ohashi] is cultivated occasionally in various parts of south-east and east Asia.14,15) Recently, the Vigna species V. mungo was found to have anti-hyperglycemic effects in diabetic rats.16) Extracts of V. angularis improved blood glucose and cholesterol in mice fed a high fat diet. In addition, the hypoglycemic effect of extracts of V. angularis were confirmed in type 2 diabetes mellitus model KK-Ay mice and a streptozotocin-induced type 1 diabetes model.17,18) Another species of Vigna, V. nakashimae, which is widely cultivated in Korea, has not been

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Abbreviations: ER, endoplasmic reticulum; HDL, high density lipoprotein; FFA, free fatty acid; PBA, phenyl butyric acid; TUDCA, tauroursodeoxycholic acid
reported to have any antihyperglycemic effects. Hence in this study, we investigated the potential beneficial effects of *V. nakashimae* on antihyperglycemia and antihyperlipidemia. We examined the inhibitory effects of *α*-glucosidase activity *in vitro*, and hypoglycemic effect in *db/db* mice.

**Materials and Methods**

*Plant*. The *Vigna nakashimae* (IT178464) germplasm was provided by the Genetic Resources Division of the National Institute of Agricultural Biotechnology of the Rural Development Administration (RDA) of South Korea, and seeds were grown in an experimental field of the Department of Functional Crop, NICS, RDA at Miryang, South Korea. Dried *V. nakashimae* was identified by one of authors (Tae Joung Ha). A voucher specimen was deposited in the National Institute of Agricultural Biotechnology.

*Chemicals*. *α*-Glucosidase (from baker's yeast), *p*-nitrophenyl *α*-d-glycopyranoside (PNP-G), acarbose, Na₂HPO₄·7H₂O, NaH₂PO₄, β-NADPH, and DMSO were obtained from Sigma Chemical (St. Louis, MO). Analytical grade ethanol and water were from J. T. Baker (Phillipsburg, NJ).

*Preparation of *Vigna nakashimae* extract*. Dried seeds of *V. nakashimae* (3.0 kg, IT178464) were pulverized with a grinder and extracted with 10 L of 80% ethanol over 3 d at room temperature. After filtration, the ethanol extracts were concentrated in vacuo at 35°C to give a brownish residue. The crude extract was then freeze-dried and ground into powder (260 g), which was stored at −70°C until it was analyzed.

*α*-Glucosidase inhibitory assay. *α*-Glucosidase activity was determined as previously described, with slight modifications. 10 μL of an ethanolic inhibitor solution was mixed with 10 μL of *α*-glucosidase (5 μg/mL) dissolved in 0.1 M phosphate buffer (pH 7.0) and 2.780 μL of 0.1 M phosphate buffer (pH 7.0) in a quartz cuvette. After 5 min of incubation at 37°C, 200 μL of 6.0 mM *p*-nitrophenyl-α-d-glycopyranoside (PNP-G) was added. The reaction solution was mixed, and enzyme activity was determined by monitoring the *p*-nitrophenol released from PNP-G at 400 nm. One unit of *α*-glucosidase was defined as the amount of enzyme that liberated 1.0 μmol of PNP per min under the assay conditions. Three concentrations (200, 300, and 400 μM) of PNP-G were selected for Dixon plots. The assay was conducted in triplicate in separate experiments. Data analysis was conducted using Sigma Plot 2000 (SPSS, Chicago, IL). The inhibitory concentration (IC₅₀) was obtained by fitting the experimental data to the logistic curve using the following equation:

\[
\text{Activity} = \frac{1}{1 + \left(\frac{\text{IC}_{50}}{C}\right)^n}
\]

The inhibition mode was analyzed using Enzyme Kinetics Module 1.0 (SPSS) equipped with Sigma Plot 2000.

**Sucrose loading test in normal ICR mice.** Normal male 6-week-old ICR mice (n = 24) were fasted overnight, after which they were divided into two groups that were treated with *V. nakashimae* extract (100 and 500 mg/kg of body weight), as well as a positive control group (acarbose, 50 mg/kg of body weight) and a control group. The mice in the *V. nakashimae* extract group were orally administered 2 g of sucrose per kg of body weight and *V. nakashimae* extract, and those in the positive control group received 2 g of sucrose per kg of body weight and acarbose orally. In the control group, the mice were orally administered the same dose of sucrose and water. The glucose levels of blood drawn from the tail vein were determined immediately upon collection at 30, 60, 90, 120, and 180 min after sucrose injection using a glucometer (Glucodr, Allmedicus, Anyang, Gyeonggi-do, South Korea).

**Administration of *V. nakashimae* extract to the *db/db* mice.** Male C57BL/KsJ-leprdb/leprdb (db/db) mice (8 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME). They were housed in a conventional state with appropriate temperature (21−23°C) and humidity (40−60%) controls under a 12 h light/12 h dark cycle, and had free access to food and water. All the groups were fed a standard AIN-76 semi-synthetic diet. They were handled in strict accordance with the Pusan National University Guidelines for the Care and Use of Laboratory animals.

After a 2-week adaptation period, the 10-week-old mice were divided into four groups (n = 7 in each group): the diabetic control (distilled water-treated) (db/db) group, the *V. nakashimae* low-dose extract group (db/db-low-dose, 100 mg/kg), and the *V. nakashimae* high-dose extract group (db/db-high-dose, 500 mg/kg). *V. nakashimae* extracts and acarbose were dissolved in distilled water and administered orally for 15 d.

**Measurement of fasting glucose and postprandial glucose levels in the db/db mice.** On day 15, blood samples were collected from the mice that had been fasted overnight. They were analyzed with a GlucoDr™ Plus glucometer (Allmedicus). In addition, postprandial blood glucose in the mice was measured at day 15. To accomplish this, the mice were fasted overnight and postprandial glucose level was determined immediately upon collection 60 min after glucose injection (0.5 g/kg of BW) intraperitoneally.

**Measurement of glycosylated hemoglobin (HbA₁c) and plasma glucagon levels.** The mice were fasted overnight, and then blood samples were taken from the inferior vena cava and glycosylated hemoglobin levels were measured by In2it™ (I) Hemoglobin A₁c Test (Bio-Rad Laboratories, Deeside, UK). Plasma glucagon levels were determined with a Mouse ELISA kit (alpco diagnostics, Salem, NH).

**Intraperitoneal glucose tolerance test (IPGTT).** Oned before sacrifice, an intraperitoneal glucose tolerance test (IPGTT) was conducted on all of the db/db mice after overnight fasting. To determine glucose tolerance, the mice were injected intraperitoneally with glucose (0.5 g/kg of BW), and the glucose concentrations of blood drawn from the tail vein were determined immediately upon collection 30, 60, and 120 min after glucose injection using a Glucometer (Glucodr, Allmedicus).

**Measurement of plasma triglyceride and HDL cholesterol levels.** The mice were fasted overnight, and then blood samples were taken from the inferior vena cava. Plasma glucagon levels were then measured a Mouse ELISA Kit (ALPCO Diagnostics). In addition, the plasma concentrations of glucose, triglyceride, and HDL-cholesterol (Asan Diagnostics, Seoul, Korea) were determined by an enzymatic method. All blood samples (plasma) obtained were centrifuged at 1,000 × g for 15 min at 4°C for biochemical analysis.

**Statistical analysis.** All data are presented as the means ± SE. Data were evaluated by one-way ANOVA, and the differences between means were determined by Duncan’s multiple-range test. All analyses were conducted using SPSS. Correlation analyses utilized Pearson’s coefficient. Values were considered statistically significant at p < 0.05.

**Results**

**Effects of *V. nakashimae* extract on inhibition of α-glucosidase activity.** We determined the inhibitory effect of *V. nakashimae* on α-glucosidases *in vitro*. As shown in Fig. 1A, the 80% ethanolic extract showed a dose-dependent inhibitory effect on α-glucosidase. As the concentration of the extract increased, enzyme activity was completely suppressed. The 50% inhibitory concentration (IC₅₀) of the extract was estimated to be 8.5 μg/mL. As compared
with acarbose, a commercial α-glucosidase inhibitor, the extract has higher inhibitory activity than acarbose. To demonstrate the α-glucosidase inhibitory activity of the extract in vivo, the blood glucose-lowering effect of the extract was evaluated in ICR mice loaded with sucrose. The blood glucose level was measured at 30-min intervals from 0 to 120 and 180 min. As shown in Fig. 1B, the extract exerted a decrease of blood glucose 30 min after sucrose loading. It reduced the postprandial hyperglycemia caused by sucrose loading to an extent less than that observed for the acarbose administered group. The difference in the glucose lowering activity of V. nakashimae in vitro and in vivo experiment might have been due to absorption of the extract into the system through the intestinal tract in vivo. Taken together, these results indicate that V. nakashimae extract has a potent inhibitory effect against α-glucosidase, and has a potential role as an antidiabetic agent.

Effects of V. nakashimae extract on blood glucose levels in the db/db mice

To examine the in vivo anti-diabetic effects of the V. nakashimae extract on diabetes, C57BL/KsJ-db/db mice were treated orally with two different concentrations of extract (100 and 500 mg/kg) every day for 2 weeks (from 10 to 12 weeks of age), and the extract effects were compared with acarbose. Fasting and postprandial blood glucose levels were measured every 5 d. Extract treatment of the db/db mice did not have any significant effect on their body weights or food intake (data not shown). However, as shown in Fig. 2, a high dose of the extract led to a significant reduction in postprandial blood glucose levels (Fig. 2A). In addition, a high dose of the extract decreased fasting blood glucose during the experimental period, whereas acarbose reduced the fasting blood glucose level 5 d after administration, but did not decrease the fasting blood glucose level at day 15 (Fig. 2B).

Effects of V. nakashimae extract on glycosylated HbA1c and glucagon in the db/db mice

We also measured blood glycosylated hemoglobin and glucagon to determine whether the extract would improve blood hyperglycemia. As shown in Fig. 3A, a high dose of the extract lowered the blood glycosylated hemoglobin level as compared to the diabetic control db/db mice. In addition, the glucagon level was also significantly lower in the high-dose extract-treated db/
Effects of V. nakashimae extract on plasma lipids in the db/db mice

Next, the effects of the extract on plasma triglycerides and free fatty acids (FFAs) levels and total cholesterol were investigated. Specifically, a dose of the extract was found to lead to a significant decrease in plasma free fatty acids and triglycerides, while it led to increased levels of HDL-cholesterol (Table 1). It is well known that lipolysis and circulating FFAs increase under insulin resistance conditions. Hence these results indicate that a decrease in plasma lipids can contribute to improvement of severe diabetes.

Effect of V. nakashimae extract on the attenuation of endoplasmic reticulum (ER) stress

It was suggested recently that ER stress plays a central role in the development of insulin resistance and diabetes by impairing insulin signaling through c-Jun NH$_2$-terminal kinase (JNK) activation. Hence we investigated whether the extract inhibits ER stress. To accomplish this, we examined the inhibitory effects on luciferase activity of the ER stress response element (ERSE)-containing reporter in HepG2 cells treated with ER stress inducer thapsigargin. While thapsigargin treatment increased ERSE-dependent luciferase activity, the extract effectively blocked thapsigargin-mediated stimulation (Fig. 5A). When ER stress indicators such as GRP78 and ATF3 were examined in thapsigargin-treated HepG2 cells, extract treatment suppressed the increase in the indicators due to thapsigargin (Fig. 5B). Taken together, these results indicate that the extract can contribute to a reduction in fasting glucose levels through attenuation of ER stress.

Discussion

Adequate control of postprandial blood glucose levels is highly important in preventing the onset of type 2 diabetes and its complications in pre-diabetic and diabetic patients. α-Glucosidase inhibitors have been the most common oral agents used for improvement of postprandial hyperglycemia since they were introduced in the early 1990s. Acarbose and voglibose are administered individually as oral hypoglycemic agents to diabetic patients.

Table 1. Effects of the Vigna nakashimae Extract on Plasma Lipid Profiles of db/db Mice

<table>
<thead>
<tr>
<th>Plasma (mmol/L)</th>
<th>FFA</th>
<th>Triglyceride</th>
<th>Total-C</th>
<th>HDL-cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.16 ± 0.03$^a$</td>
<td>6.72 ± 0.53$^a$</td>
<td>3.07 ± 0.13NS</td>
<td>0.98 ± 0.09$^b$</td>
</tr>
<tr>
<td>Acarbose</td>
<td>0.95 ± 0.08$^b$</td>
<td>7.49 ± 0.55$^b$</td>
<td>2.80 ± 0.08</td>
<td>1.07 ± 0.05$^c$</td>
</tr>
<tr>
<td>Low dose</td>
<td>0.91 ± 0.02$^b$</td>
<td>5.58 ± 0.91$^c$</td>
<td>3.09 ± 0.33</td>
<td>1.07 ± 0.23$^c$</td>
</tr>
<tr>
<td>High dose</td>
<td>0.94 ± 0.06$^b$</td>
<td>3.17 ± 0.40$^d$</td>
<td>2.70 ± 0.13</td>
<td>1.12 ± 0.20$^b$</td>
</tr>
</tbody>
</table>

$^{ab}$Means not sharing a common superscript indicate a significant difference ($p < 0.05$) between groups after one-way ANOVA and Duncan’s multiple-range test. mean ± SE (n = 7). FFAs, free fatty acids.

Effects of V. nakashimae extract on glucose tolerance in the db/db mice

To assess glucose homeostasis in the db/db mice treated with the extract, glucose tolerance was measured before the end of the experiment. The blood glucose change rate did not differ significantly between the extract-treated group and the diabetic control db/db mice for up to 60 min (Fig. 4). However, at 120 min after glucose loading, the extract induced a significant reduction in blood glucose levels in a fashion similar to acarbose, used as a positive control, as compared with the diabetic control group. This indicates that the extract treatment affected the regulation of the postprandial glucose level in the db/db mice.

Fig. 3. Effects of V. nakashimae Extract on Glycated Hemoglobin (hemoglobin A1c, HbA$_1c$) (A) and Plasma Glucagon (B) in C57BL/ KsJ-db/db Mice.

After oral administration for 15 d, plasma glycated hemoglobin and glucagon levels were determined. Values are expressed as mean ± SE. (n = 7). $^{ab}$Means not sharing a common letter are significantly different ($p < 0.05$).

Fig. 4. Effects of V. nakashimae Extract on the Intraperitoneal Glucose Tolerance Test (IPGTT) in C57BL/ KsJ-db/db Mice.

One d before sacrifice, the mice were injected intraperitoneally with glucose (0.5 g/kg BW) after a 12-h fast. The blood glucose concentration was measured at the indicated times, and is presented as a percentage of the glucose injection zero time. Values are expressed as mean ± SE, (n = 7). $^{ab}$Means not sharing a common letter are significantly different ($p < 0.05$). NS, not significant.

db mice (Fig. 3B). These results suggest that the extract can improve hyperglycemia in db/db mice.

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efficiency values of the V. nakashimae in improvement of hyperglycemia and diabetes symptoms. It suggests that the extract might have beneficial antihyperglycemic effects in diabetic animals through inhibition of α-glucosidase. Therefore, we examined the antihyperglycemic effect and antidyshlipidemia in db/db mice. The extract showed potent in vitro inhibition of α-glucosidase, which prompted us to determine whether the extract also inhibits α-glucosidase in vivo, thereby limiting or delaying starch digestion and absorption, and subsequently reducing the postprandial glycemic response. The results of a sucrose loading test indicated that the extract reduced postprandial increases in the blood glucose level, which suggests that the extract might have beneficial antihyperglycemic effects in diabetic animals through inhibition of α-glucosidase. Therefore, we examined the improvement of hyperglycemia and diabetes symptoms in db/db mice. The food intake, body weight, and feed efficiency values of the V. nakashimae extract did not differ significantly from the control group. An ethanol extract of V. nakashimae led to significant decreases in both the postprandial and the fasting blood glucose level. It has been suggested that reducing glucose toxicity by decreasing postprandial glucose elevation results in improved overall glycemic control. The decreased postprandial blood glucose elevation in response to the extract might have been due to α-glucosidase inhibitory action resulting in reduced fasting blood glucose levels.

It was reported recently that ER stress induces the development of type 2 diabetes because it activates JNK, which induces insulin resistance in the liver and skeletal muscle and inhibits beta-cell function. Hence agents that alleviate ER stress might act as potent anti-diabetic agents with the potential for application in the treatment of type 2 diabetes. Chemical and biological compounds such as macelignan, chromium-phenylalanine, phenyl butyric acid (PBA) and tauroursodeoxycholic acid (TUDCA) and molecular chaperones have been found to inhibit ER stress and enhance insulin sensitivity, thereby normalizing hyperglycemia. In the present study, V. nakashimae extract was found to alleviate ER stress based on the results of a ERSE reporter assay and measurement of ER stress indicator proteins in HepG2 cells. The V. nakashimae extract efficiently suppressed ERSE-dependent transactivation in thapsigargin-treated HepG2 and the expression of ER stress marker proteins. Thus, the capacity of the extract to inhibit ER stress can contribute to decreased fasting blood glucose levels through amelioration of insulin sensitivity.

Furthermore, the V. nakashimae extract was effective at reducing plasma triglyceride and tended to increase HDL cholesterol. It has also been reported that long-term consumption of acarbose reduced blood cholesterol and triglycerides levels in an animal model of diabetes. Additionally, it has been suggested that acarbose improves blood lipid profiles by increasing insulin sensitivity. Zavaroni and Reaven suggested that chronic α-glucosidase inhibitors lower VLDL-triglyceride secretion, resulting in improved hypertriglyceridemia and hypercholesterolemia. Similarly to the above α-glucosidase inhibitors, V. nakashimae extract improved the lipid profiles in db/db mice.

Even though the components that exert anti-hyperglycemic and anti-hyperlipidemic effects have not yet been characterized, HPLC analysis of V. nakashimae and V. angularis extracts showed different chromatogram profiles (data not shown), suggesting that different bio-active components might have contributed to the antidiabetic effects of the V. nakashimae extract. Further study should be done to characterize the antidiabetic components of V. nakashimae extract.
In conclusion, *V. nakashimae* extract exerted a potential antidiabetic effect by suppressing postprandial and fasting blood glucose through inhibition of intestinal α-glucosidase and ER stress and blood hyperlipidemia. Hence *V. nakashimae* might be a useful natural antidiabetic agent to reduce the risk of diabetic complications.

**Acknowledgments**

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**References**