Anti-diabetic Effects of Adlay Protein in Type 2 Diabetic db/db Mice

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We examined the effects of adlay protein concentrates (AP) on lipid metabolism and on in vivo oxidative stress in type 2 diabetic db/db mice. Mice were fed an AIN-93G diet containing either adlay grains (AG) or AP for 21 days. Plasma parameters, such as total cholesterol, arteriosclerotic index and thiobarbituric acid reactive substances (TBARS) concentration in the diabetic AP-fed group were lower than those in the diabetic control (AIN-93G) group. Concentrations of hepatic parameters, such as total cholesterol, triglyceride and TBARS levels, in the AP-fed group were lower than those in the diabetic control group. A pronounced improvement in lipid metabolism of the AP-fed group was observed when compared with the AG-fed group. It was deduced that excretion of bile acids and cholesterol in the feces of mice fed the AP diet abundant in resistant protein contributed to the suppression of plasma and hepatic cholesterol concentration in diabetic mice.

Keywords: adlay grains, adlay protein, type 2 diabetic mice, lipid regulation, oxidative stress

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Introduction

Adlay (Coix lacryma-jobi L. var. ma-yuen Stapf.) originates from Southeast Asia, and is mainly cultivated in India, China and Japan. Although adlay plants were occasionally used as a forage crop, the current importance of the crop is now the usage of its seeds as an herbal medicine (yi yi ren). These seeds are traditionally used in China and Japan to treat rheumatism, warts, neuralgia and problems associated with the female endocrine system (Kim et al., 2004). Recent studies have also demonstrated that adlay extracts have anti-obesity (Kim et al., 2007) and hypolipidemic effects (Kim et al., 2004). In addition, coixenolide (Tanimura, 1961), which has anti-tumor activity (Ukita and Tanimura, 1961), and coixol, which has an analgesic effect (Koyama, 1955), have been identified as bioactive substances in adlay grains (AG).

AG is rich in protein, lipid, vitamin B1 and B2, and iron; whole grains have particularly high protein contents (16.5%) (Park et al., 1988) when compared with other cereals, such as wheat, rice and buckwheat. The major protein in AG is coixin, which is an alcohol-soluble protein belonging to the prolamine group. Coixin is composed of the following five classes of polypeptides: 27 kDa (C1), 25 kDa (C2), 22 kDa (C3), 17 kDa (C4), and 15 kDa (C5) (Leite et al., 1991).

Moreover, the importance of AG as a functional food ingredient in products such as tea and various processed foods is increasing. Therefore, further investigations of the in vivo activities induced by oral administration of AG are necessary.

The increase in the incidence of diabetes mellitus, which is also a risk factor for metabolic syndrome, is a worldwide issue. In Japan, the number of people who are strongly suspected to have diabetes and who possibly have diabetes was 8.2 and 10.5 million, respectively, according to data from the National Health and Nutrition Survey Japan 2006i). From the perspective of reducing medical costs, products that help prevent the onset of and complications resulting from type 2 diabetes mellitus are of great interest; for example, protein concentrates from millets such as foxtail millet, proso millet and Japanese barnyard millet have hypoglycemic and hypolipidemic effects (Choi et al., 2005, Park et al., 2008, Nishizawa et al., 2009).

We recently investigated the in vivo effects of oral administration of millet grains in type 2 diabetic mice, and the results suggested that adlay and Japanese barnyard millet were effective in suppressing hepatic cholesterol and triglyceride content (Watanabe et al., 2011). In the present study, we investigated the effects of adlay protein concentrates (AP) on lipid metabolism and on in vivo oxidative stress in type 2 diabetic mice, as compared to the effects of AG.
Materials and Methods

Preparation of AP and diets based on AIN-93G containing AG or AP  Adlay (cultivar, Hatojiro) was harvested at the National Agricultural Research Center for Tohoku Region in 2009, and the grains were ground to a powder in a vibrating sample mill. Protein concentrates from the grains were prepared according to Nishizawa’s method (1990) as follows. Two liters of distilled water was added to 200 g of adlay powder, and the mixture was agitated. α-Amylase (170 mg, Uniase BM-8; Yakult, Tokyo, Japan) was added to the mixture and the temperature raised to 90°C with occasional agitation. The temperature was maintained for 30 min to allow gelatinization and was subsequently reduced to 60°C. Glucoamylase (600 mg, Uniase 30; Yakult, Tokyo, Japan) was added to the mixture, and the temperature was maintained at 60°C for 24 h with gentle agitation. The mixture was centrifuged at 19,000 × g for 10 min and the precipitate was collected. Precipitates were freeze-dried and the dried powder was defatted four times with n-hexane. The whole procedure was performed 10 times and 470 g of AP were prepared. The general composite formation and amino acid composition of AP (g/100 g dry weight) and AG are shown in Tables 1 and 2, respectively. The protein content increased from 18.2% in AG to 64.4% in AP, i.e., 3.5-fold that of AG, with a decrease in carbohydrate content from 55.2% in AG to 13.7% in AP. In addition, the resistant protein content of AP, as determined by treatment with pepsin and pancreatin according to Iwami’s method (2002), was 32%.

Table 3 lists the composition of experimental diets for mice. The AP or AG diet contained 20% AP or AG powder respectively, and the contents of casein (source of protein) and cellulose (source of dietary fiber) were adjusted based on the composition of AP or AG.

Animals  Male diabetic db/db (C57BL/KsJ-lepr<sup>db</sup>/lepr<sup>db</sup>) and nondiabetic db/+ (C57BL/KsJ-m/+lepr<sup>db</sup>) mice were purchased from Japan Clea Inc. (Tokyo, Japan). Mice were housed in plastic cages in a temperature-controlled room (23 ± 1°C) under light and dark conditions (12 h each) and had free access to water and feed (CE-2; Japan CLEA Inc.) until the experiment began. At 7 weeks of age, mice were randomly divided into four groups of eight mice each. Diabetic mice in the adlay grains group (DG) were fed a 20% AG diet based on AIN-93G (Table 3). Diabetic mice in the adlay protein concentrates group (DP) were fed a 20% AP diet based on AIN-93G. Diabetic and nondiabetic mice in the control groups that were assigned into two groups as DC and NC, respectively, were fed AIN-93G diet. After 21 days of treatment, the mice were fasted for 16 h, and their blood was collected in heparinized tubes from the heart under anesthesia by intraperitoneal injection of sodium pentobarbital (40 mg/
kg body weight) before being sacrificed. Thereafter, the liver and epididymal adipose tissues of the mice were sampled and the hemoglobin A1c (HbA1c) concentration in whole blood was measured using an immunoassay system (DCA 2000; Siemens, Munich, Germany). Sampled tissues were immediately frozen in liquid nitrogen and stored at −80°C until use. In addition, feces were collected over 24 h on 6 days before sacrificing the mice. Mice were treated in accordance with the Guide for the Care and Use of Experimental Animals (National Agricultural Research Center for Tohoku Region).

**Extraction of lipids from liver tissues** Total lipids in the liver tissues were extracted with chloroform-methanol (2:1, v/v) solvent according to Folch’s method (Folch et al., 1957). After removing the solvent from the extracts, the residue was dissolved in 2-propanol containing 10% Triton X-100. This crude lipid solution was used for measurement of lipids.

**Measurement of plasma glucose** After preparation of plasma by centrifuging the collected blood at 11,000 × g for 5 min at 4°C, plasma glucose was measured using a Glucose C-II Test Wako kit (Wako Pure Chemical Industries Ltd., Osaka, Japan) according to the manufacturer’s instructions.

**Measurement of lipids** Concentrations of total cholesterol, HDL cholesterol, and triglycerides were measured using the Cholesterol E Test, HDL cholesterol E Test and triglyceride E Test kits (Wako Pure Chemical Ltd.), respectively.

**Assay for TBARS in liver tissues and plasma** The concentration of TBARS in liver tissue homogenates was analyzed using Kikugawa’s method (Kikugawa et al., 1992). Plasma TBARS concentration was analyzed according to Yagi’s method (Yagi, 1976).

**Measurement of protein content in liver homogenates** Protein concentration in liver homogenates was measured using a Protein Quantification Kit-Rapid (Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer’s instructions.

**Measurement of bile acids in liver homogenates** Bile acid was extracted from liver tissues by the solid extraction method according to Sakamura’s method (Sakamura et al., 1993). Briefly, bile acid was extracted from 100 mg of the liver tissues using ethanol at 70°C for 1 h, and was then passed through the Sep-pak C18 cartridge (Waters, Milford, MA, USA). After washing the column with 5 mL of water, bile acid was eluted with 5 mL of methanol. Bile acid level was determined using the Total Bile Acid Test Wako kit (Wako Pure Chemical Ltd.) according to the manufacturer’s instruction.

**Extraction of total RNA from liver tissues and synthesis of cDNA** Total RNA was extracted from 30 mg of liver tissues using an Absolutely RNA Miniprep kit (Stratagene, La Jolla, CA, USA) according to the manufacturer’s instructions. Level and purity of RNA were analyzed by UV ratio (260/280 nm), followed by electrophoresis. cDNA was synthesized using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions.

**Measurement of gene expression of enzymes and sterol transporters involved in lipid metabolism by quantitative real-time PCR analysis** Gene expression of enzymes and sterol transporters involved in lipid metabolism using cDNA templates were analyzed by quantitative real-time PCR analysis with an Applied Biosystems 7500 real-time PCR system using TaqMan primer-probe sets and TaqMan® Universal PCR Master Mix (Applied Biosystems). The analyzed genes and their assay ID for TaqMan primer-probe sets (TaqMan Gene Expression Assay, Applied Biosystems) were as follows: 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoAR), regulates hepatic cholesterol synthesis, assay ID Mm01282499_m1; cholesterol 7a-hydroxylase (CYP7A1), catalyzes bile acid synthesis, assay ID Mm00448415_m1; diacylglycerol acyltransferase (DGAT), catalyzes triglyceride synthesis, assay ID Mm00515643_m1; hepatic triglyceride lipase (HTGL), catalyzes conversion of intermediate-density lipoprotein (IDL) to low-density lipoprotein by hydrolyzing triglycerides in IDL, assay ID Mm00433975_m1; acetyl-CoA carboxylase (ACC), catalyzes malonyl-CoA production from acetyl-CoA in fatty acid synthesis, assay ID Mm01204667_m1; fatty acid synthase (FAS), regulates fatty acid synthesis, assay ID Mm06623191_m1; carnitine palmitoyltransferase I (CPT I), catalyzes acylcarnitine production from acyl-CoA and L-carnitine in β-oxidation pathway, assay ID Mm00550438_m1; ATP-binding cassette sub-family G member 5 (ABCG5), mediates the excretion of cholesterol, assay ID Mm00446241_m1; and ATP-binding cassette sub-family G member 8 (ABCG8), mediates the excretion of cholesterol, assay ID Mm00445970_m1. Expression of target genes was normalized using an 18S rRNA control (Applied Biosystems).

**Measurement of bile acids and total cholesterol in feces** Mice feces were collected, freeze-dried and ground to powder using a vibrating mill. Bile acid was extracted from the powder with ethanol at 70°C for 1 h, and cholesterol was extracted by Folch’s method. Bile acid and total cholesterol levels were determined using the Total Bile Acid Test Wako kit and Cholesterol E Test, respectively, (Wako Pure Chemical Ltd.) according to the manufacturer’s instructions.

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE)** In order to confirm the molecular characteristics of AP, a protein extract was prepared using SDS-urea solution. SDS-PAGE was performed according to Laemmli’s method.
plasma glucose, there were no significant differences between the DC, DG and DP groups. For lipid metabolism, the total plasma cholesterol content in the DP group was lower than that in the DC group. Plasma HDL cholesterol content was not significantly different among the diabetic mouse groups. Consequently, the arteriosclerotic index, calculated from the total cholesterol and HDL cholesterol contents, in the DP group was significantly lower than that in the DC group.

Statistics  Statistically significant differences between groups were evaluated by one-way analysis of variance (ANOVA) followed by a Tukey test using Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). Differences at \( P < 0.05 \) were considered significant.

Results  Figure 1 shows the SDS-PAGE profile of proteins from AP. The major molecular classes observed in lane 2 were 27 kDa, 25 kDa, 22 kDa, 17 kDa and 15 kDa. This result demonstrates that the major protein in AP is a prolamine, by comparison with that of SDS-PAGE analysis for coixin, which belongs to the prolamine group, from adlay (Leite et al., 1990). AP contained proteins of more than 30 kDa, with the exception of prolamine.

Table 4 shows the food intake, final body weight, body weight gain, liver weight and epididymal adipose tissue weight of the \( \text{db}^+/+ \) (NC group) and \( \text{db}/\text{db} \) (DC, DG and DP groups) mice. Although the values of all these parameters in the diabetic mice (DC, DG and DP groups) were higher than those in the NC group, there were no significant differences among the diabetic mouse groups.

Table 5 shows the blood and plasma parameters. In parameters for carbohydrate metabolism, such as HbA1c and plasma glucose, there were no significant differences between the DC, DG and DP groups. For lipid metabolism, the total plasma cholesterol content in the DP group was lower than that in the DC group. Plasma HDL cholesterol content was not significantly different among the diabetic mouse groups. Consequently, the arteriosclerotic index, calculated from the total cholesterol and HDL cholesterol contents, in the DP group was significantly lower than that in the DC group.

**Table 4.** Food intake, final body weight, body weight gain, liver weight, and epididymal adipose tissue weight of \( \text{db}^+/+ \) (NC group) and \( \text{db}/\text{db} \) (DC, DG and DP groups) mice.

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>NC</th>
<th>DC</th>
<th>DG</th>
<th>DP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food intake (g/d)</td>
<td>3.7 ± 0.2 (^a)</td>
<td>5.1 ± 0.2 (^b)</td>
<td>5.3 ± 0.2 (^b)</td>
<td>5.2 ± 0.2 (^b)</td>
</tr>
<tr>
<td>Final body-wt (g)</td>
<td>27.2 ± 0.4 (^a)</td>
<td>38.7 ± 0.7 (^b)</td>
<td>39.1 ± 0.8 (^b)</td>
<td>39.4 ± 0.5 (^b)</td>
</tr>
<tr>
<td>Body-wt. gain (g/21 d)</td>
<td>2.7 ± 0.3 (^a)</td>
<td>8.6 ± 0.5 (^b)</td>
<td>9.2 ± 0.9 (^b)</td>
<td>9.3 ± 0.4 (^b)</td>
</tr>
<tr>
<td>Liver-wt. (g/100 g body wt.)</td>
<td>4.4 ± 0.1 (^a)</td>
<td>5.1 ± 0.2 (^b)</td>
<td>5.0 ± 0.1 (^b)</td>
<td>5.5 ± 0.1 (^b)</td>
</tr>
<tr>
<td>Epididymal adipose tissue-wt. (g/100 g body wt.)</td>
<td>1.0 ± 0.0 (^a)</td>
<td>2.4 ± 0.1 (^b)</td>
<td>2.6 ± 0.1 (^b)</td>
<td>2.5 ± 0.1 (^b)</td>
</tr>
</tbody>
</table>

Values are means ± SE of 8 mice. Different superscript letters indicate significant differences (\( P < 0.05 \)). NC, nondiabetic control diet (AIN-93G) group; DC, diabetic control diet (AIN-93G) group; DG, diabetic adlay grain (AG) diet group; DP, diabetic adlay protein concentrate (AP) diet group.

**Table 5.** Blood and plasma parameters of \( \text{db}^+/+ \) (NC group) and \( \text{db}/\text{db} \) (DC, DG and DP groups) mice.

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>NC</th>
<th>DC</th>
<th>DG</th>
<th>DP</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA1c (%)</td>
<td>3.9 ± 0.1 (^a)</td>
<td>8.7 ± 0.2 (^b)</td>
<td>8.7 ± 0.3 (^b)</td>
<td>8.0 ± 0.2 (^b)</td>
</tr>
<tr>
<td>Plasma glucose (mg/dL)</td>
<td>104 ± 2 (^a)</td>
<td>641 ± 22 (^b)</td>
<td>634 ± 29 (^b)</td>
<td>621 ± 18 (^b)</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>84 ± 4 (^a)</td>
<td>181 ± 4 (^c)</td>
<td>172 ± 6 (^c)</td>
<td>153 ± 3 (^b)</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>56 ± 2 (^a)</td>
<td>103 ± 2 (^b)</td>
<td>103 ± 4 (^b)</td>
<td>96 ± 2 (^b)</td>
</tr>
<tr>
<td>Arteriosclerotic index</td>
<td>0.49 ± 0.03 (^a)</td>
<td>0.76 ± 0.04 (^c)</td>
<td>0.68 ± 0.03 (^c)</td>
<td>0.60 ± 0.03 (^b)</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>63 ± 4 (^a)</td>
<td>85 ± 4 (^b)</td>
<td>64 ± 4 (^b)</td>
<td>54 ± 2 (^b)</td>
</tr>
</tbody>
</table>

Values are means ± SE of 8 mice. Different superscript letters indicate significant differences (\( P < 0.05 \)). NC, nondiabetic control diet (AIN-93G) group; DC, diabetic control diet (AIN-93G) group; DG, diabetic adlay grain (AG) diet group; DP, diabetic adlay protein concentrate (AP) diet group. " analyzed with whole blood. " Arteriosclerotic index: (total cholesterol − HDL cholesterol)/HDL cholesterol.
group. Furthermore, triglyceride contents of the DG and DP groups were significantly lower than those of the DC group.

The total cholesterol content in the liver tissues was significantly lower in the DG and DP groups than in the DC group (Table 6). However, there were no significant differences in triglyceride content among the diabetic mouse groups. Bile acid in the liver tissues did not differ among the mouse groups.

Figure 2 shows the bile acid and cholesterol contents in the feces. Bile acid content in the DG and DP groups was significantly higher than in the NC and DC groups, and that in DP group was higher than that in the DG group. The total cholesterol content in the DG and DP groups was higher than that in the DC group, and that in the DP group was higher than the total cholesterol content in the DG group.

Table 7 shows the gene expression of hepatic enzymes and sterol transporters involved in lipid metabolism. There were no significant differences in the gene expression of any enzymes and transporters involved in lipid regulation between the DP group and DC group.

The TBARS concentration in the plasma and liver tissues (Fig. 3) in the diabetic mouse groups was significantly higher than that in the NC group, thus suggesting that in vivo oxidative stress was elevated in the diabetic mouse groups. Among

### Table 6. Hepatic lipids of db/+ (NC group) and db/db (DC, DG, and DP groups) mice.

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>NC</th>
<th>DC</th>
<th>DG</th>
<th>DP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/g tissue)</td>
<td>4.5 ± 0.3 a</td>
<td>9.8 ± 0.5 c</td>
<td>6.8 ± 0.2 b</td>
<td>7.3 ± 0.4 b</td>
</tr>
<tr>
<td>Triglyceride (mg/g tissue)</td>
<td>24 ± 1 a</td>
<td>96 ± 7 b</td>
<td>97 ± 8 b</td>
<td>114 ± 8 b</td>
</tr>
<tr>
<td>Bile acid (μmol/g tissue)</td>
<td>0.095 ± 0.002</td>
<td>0.097 ± 0.007</td>
<td>0.101 ± 0.004</td>
<td>0.101 ± 0.004</td>
</tr>
</tbody>
</table>

Values are means ± SE of 8 mice. Different superscript letters indicate significant differences (P < 0.05). NC, nondiabetic control diet (AIN-93G) group; DC, diabetic control diet (AIN-93G) group; DG, diabetic adlay grain (AG) diet group; DP, diabetic adlay protein concentrate (AP) diet group.

### Table 7. Levels of mRNA in hepatic enzymes of lipid metabolism and sterol transporters in db/+ (NC group) and db/db (DC, DG and DP groups) mice.

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>NC</th>
<th>DC</th>
<th>DG</th>
<th>DP</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMG-CoAR</td>
<td>1.00 ± 0.04 a</td>
<td>1.47 ± 0.19 ab</td>
<td>1.52 ± 0.09 ab</td>
<td>1.81 ± 0.16 b</td>
</tr>
<tr>
<td>CYP7A1</td>
<td>1.00 ± 0.09 a</td>
<td>1.73 ± 0.15 ab</td>
<td>2.69 ± 0.43 b</td>
<td>1.83 ± 0.23 ab</td>
</tr>
<tr>
<td>DGAT</td>
<td>1.00 ± 0.12</td>
<td>1.20 ± 0.17</td>
<td>0.99 ± 0.20</td>
<td>1.19 ± 0.20</td>
</tr>
<tr>
<td>HTGL</td>
<td>1.00 ± 0.10</td>
<td>1.30 ± 0.13</td>
<td>1.32 ± 0.14</td>
<td>1.37 ± 0.13</td>
</tr>
<tr>
<td>ACC</td>
<td>1.00 ± 0.12</td>
<td>1.11 ± 0.21</td>
<td>0.78 ± 0.13</td>
<td>0.90 ± 0.11</td>
</tr>
<tr>
<td>FAS</td>
<td>1.00 ± 0.16 a</td>
<td>4.49 ± 1.19 b</td>
<td>2.97 ± 0.55 a</td>
<td>4.08 ± 0.56 b</td>
</tr>
<tr>
<td>CPT I</td>
<td>1.00 ± 0.10 a</td>
<td>0.88 ± 0.12 a</td>
<td>0.65 ± 0.04 a</td>
<td>0.76 ± 0.07 a</td>
</tr>
<tr>
<td>ABCG5</td>
<td>1.00 ± 0.11 a</td>
<td>1.62 ± 0.25 ab</td>
<td>1.35 ± 0.15 ab</td>
<td>2.05 ± 0.29 b</td>
</tr>
<tr>
<td>ABCG8</td>
<td>1.00 ± 0.08</td>
<td>1.19 ± 0.14</td>
<td>1.11 ± 0.16</td>
<td>1.57 ± 0.27</td>
</tr>
</tbody>
</table>

Values are means ± SE of 8 mice. Different superscript letters indicate significant differences (P < 0.05). NC, nondiabetic control diet (AIN-93G) group; DC, diabetic control diet (AIN-93G) group; DG, diabetic adlay grain (AG) diet group; DP, diabetic adlay protein concentrate (AP) diet group. HMG-CoAR, 3-hydroxy-3-methylglutaryl-CoA reductase; CYP7A1, cholesterol 7-α-hydroxylase; DGAT, diacylglycerol acyltransferase; HTGL, hepatic triglyceride lipase; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; CPT I, carnitine palmitoyltransferase I; ABCG5, ATP-binding cassette sub-family G member 5; ABCG8, ATP-binding cassette sub-family G member 8.
with its high prolamine content, may be useful for improvement in cholesterol metabolism. Although a 20% adlay or Japanese barnyard millet diet improved lipid metabolism by decreasing total cholesterol and arteriosclerotic index in the diabetic mice, the daily administration of a 20% proso millet diet had no effect on lipid metabolism in db/db mice (Watanabe et al., 2011). Further investigation of the differences in content and characteristics of the prolamine fraction in proso millet, adlay and Japanese barnyard millet is necessary to elucidate the differences in the effects of their grains and proteins on plasma cholesterol metabolism in diabetic mice.

In this study, the AP diet had a significant effect, decreasing the total cholesterol content of plasma. Both the AG and AP diets lowered the hepatic cholesterol content in db/db mice. Consequently, AP suppressed the cholesterol content in both plasma and liver tissues in diabetic mice. The lowering of cholesterol levels is likely due to the synthesis of bile acids in liver tissues along with the acceleration in excretion of bile acids and cholesterol into feces. In recent studies, a buckwheat sprout diet (20%, w/w) exhibited cholesterol-lowering effects with an increase in bile acid excretion in the feces and an increase in the expression of the hepatic gene CYP7A1 (Watanabe and Ayugase, 2010).

In this experiment, the AG and AP diets significantly increased the excretion of bile acid and cholesterol in the feces. Moreover, the effects of AP on excretion were significantly higher than those of AG. However, there were no differences in the expression of CYP7A1 in the AG-diet, AP-diet and diabetic control groups. Because inhibitors of bile acid absorption in the small intestine are associated with the upregulation of CYP7A1 expression (Chen et al., 2008), the enhancement of bile acid excretion by AP would be attributable to its inhibitor effect of bile acid absorption.

On the other hand, Tomotake et al. (2000) suggested that the cholesterol-lowering effect of buckwheat protein (BWP) in hamsters fed diets with 5g/kg cholesterol is mediated by its influence on fecal excretion of bile acid and neutral sterols. They concluded that BWP has dietary fiber-like properties by virtue of its lower digestibility and proposed that BWP is a “resistant protein (RP)”. Whereas the RP content in AP is 32%, which is higher than that in the high molecular weight (HMF) fraction (25%) of soybean (Iwami et al., 2002). The reduction of hepatic cholesterol content in rats along with excretion of bile acid and acidic or neutral steroid excretion by 27% (HMF)-feed and 20% RP-feed was reported to be attributable to the binding activity of RP with bile acid. These results strongly suggested that high contents of RP in AP are effective on the fecal excretion of bile acid and cholesterol. For clarification of the bile acid absorption inhibitor activity of AP, measurement of binding activity of RP in AP with bile

Discussion

Daily oral administration of the AG and AP diet improved lipid metabolism in type 2 diabetic db/db mice. Improvements such as decreases in plasma total cholesterol and arteriosclerotic index, and increases in bile acid and cholesterol in the feces in mice fed the AP diet were marked when compared to mice fed the AG diet.

SDS-PAGE analysis showed that the major protein fraction of AP is a prolamine, namely coixin. The major protein fractions in other millets (proso millet, foxtail millet, and Japanese barnyard millet) are also prolamine (extracted with 70% (v/v) isopropyl alcohol) and prolamine-like proteins (extracted with 70% isopropyl alcohol containing 0.6% 2-mercaptoethanol), accounting for 63.3% to 80% of total proteins in their grains (Serna-Saldivar et al., 1991; Dendy 1995; Nishizawa and Fudamoto 1995). Moreover, the protein concentrate from proso millet was reported to have a positive effect in lipid metabolism by increasing plasma HDL-cholesterol (Nishizawa et al., 1990). These results raise the possibility that the protein fraction from millets, with its high prolamine content, may be useful for improvement in cholesterol metabolism.
acid is necessary.

The activity of CYP7A1 is regulated by a negative feedback system based on hepatic bile acid content. However, hepatic bile acid content did not differ among the mice groups after 21 days. Because the feces of the mice were collected a week before collection of the liver, bile acids that had increased in the liver in the AP-diet group may have suppressed the expression of hepatic CYP7A1. The simultaneous measurement of enzyme activity and/or protein content of CYP7A1 in the liver tissues, along with the analysis of gene expression, is necessary to clarify the effects of AP on cholesterol regulation. Although the results of the present study indicated that AP contributes to the effects of AG in lipid metabolism, further study is necessary to elucidate these effects.

AP suppressed an increase in TBARS concentration in the plasma and liver tissues of diabetic mice. Hyperglycemia in diabetes mellitus induces the production of reactive oxygen species (ROS) (Wolf et al., 1991). Hence, the suppression of increasing blood glucose levels would be important in reducing in vivo oxidative stress. HbA1c and plasma glucose levels in the DP group were not different from the DC group, thus suggesting that the AP diet had no significant effects on hyperglycemia in the diabetic mice. On the other hand, protein is the major component in AP (65%), and most water-soluble phenolic compounds were removed by enzyme treatment during AP preparation. Therefore, it has been suggested that the suppression of TBARS content in the plasma and liver tissues is due to the protein in AP.

Peptides originating from plants, such as soybean protein (Chen et al., 1995), wheat gluten (Suetsuna and Chen 2002) and rice bran globulin (Adebiyi et al., 2008), are reported to exhibit antioxidant activity. Marcuse (1960) demonstrated that amino acids such as histidine, lysine and tryptophan were antioxidants in herring oil emulsions. In addition, Chen et al., (1995) showed that antioxidant peptides from soybean protein (β-conglycinin) contained proline, histidine and tyrosine in the sequences along with hydrophobic amino acids such as valine and leucine at the N-terminal position. AP contained substantial amount of antioxidant amino acids such as leucine (8.92%), proline (5.14%), valine (3.08%), tyrosine (2.47%) and histidine (1.25%), thus suggesting that the peptides produced by enzymatic hydrolysis of orally administered AP exhibit in vivo antioxidant activity. Therefore, suppression of TBARS content in the plasma and liver tissues by the AP diet may be attributable to the antioxidant activity of peptides derived from AP. Further study is necessary to fully elucidate this issue.

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