JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 4113–4119 4113
DOI:10.1021/jf9036672

Semipurified Fractions from the Submerged-Culture Broth of *Agaricus blazei* Murill Reduce Blood Glucose Levels in Streptozotocin-Induced Diabetic Rats

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Hypoglycemic action of semipurified fractions from hot-water extracts of the submerged-culture broth of *Agaricus blazei* Murill was examined in streptozotocin (60 mg/kg, intraperitoneal)-induced diabetic male Sprague−Dawley rats, relative to the diabetes drug metformin. The hot-water extract, treated with ethanol to remove β-glucans and glycoproteins, was freeze-dried, and fractionated into hexane, chloroform, ethyl acetate (EA), and butanol fractions. The EA fraction (EAF; 200 mg/kg body weight) reduced ($p < 0.05$) the blood glucose level in the oral glucose tolerance test, relative to the other fractions and control. In a 14 day-treatment study, diabetic rats treated with the EAF displayed a suppressed blood glucose level and elevated plasma insulin and glucose transport-4 proteins; the reactions occurred in a dose-dependent manner (200 and 400 mg/kg body weight) compared to those in control animals. The EAF reduced the levels of triglyceride and cholesterol in plasma, the activity of glutamate−oxaloacetate transaminase and glutamate−pyruvate transaminase in blood, and the content of thiobarbituric acid reactive substance in the liver and kidney. The hypoglycemic efficacy of the EAF (400 mg/kg body weight) was similar to that of metformin (500 mg/kg body weight). The EAF contained substantial amounts of isoflavonoids including genistein, genistin, daidzein, and daidzin, which could have contributed to the fraction's hypoglycemic action. These results indicate that the hot-water extract of the submerged-culture broth of *Agaricus blazei* contains an EAF having potent hypoglycemic action, which could be useful in the treatment of diabetes mellitus.

KEYWORDS: ***Agaricus blazei*** **Murill; blood glucose; diabetes; hypoglycemia; serum insulin; streptozotocin**

INTRODUCTION

Diabetes mellitus (DM) is a condition characterized by hyperglycemia resulting from the body's inability to use blood glucose for energy and is categorized as insulin-dependent (type 1) and noninsulin dependent (type 2) (*1*). Many edible mushrooms and traditional plants have been widely screened for use as remedies or natural products for the control of diabetes (*2*−*5*). Such a screening might reveal effective dietary adjuncts for the treatment of DM or the discovery of natural products with promise as antidiabetic drugs. For such a purpose, fruit bodies and culture broths of some species of edible mushrooms, including *Agaricus blazei*, *Inonotus obliquus*, *Lentinus edodes*, and *Granola frondosa* have been screened for the reduction of blood glucose levels in human and animal models (*5*−*11*). The β-glucans and glycoproteins are the only hypoglycemic substances isolated from fruit bodies and culture broths of mushrooms so far (*6*−*11*).

A. blazei is a common mushroom in South America and Asia, and has been widely used in traditional medicine as a remedy for certain types of cancers and diabetes (*12*−*14*). In Asia, including the Republic of Korea, fruiting bodies of *A. blazei* have a considerable reputation as a potent remedy for DM (*15*). This use has been hindered by the extended time required (> 6 months) to grow the fruiting bodies. As an alternative strategy, submerged-liquid culturing of *A. blazei* has been explored as a means of obtaining an ample amount of mycelia and/or culture broth (*16*−*18*). This strategy has merit, given that fruiting body, mycelium, and liquid broth of *A. blazei* are comparable in their anticarcinogenic action and some other beneficial biological activities (*5*). A preliminary study in our laboratory showed that dried culture broth from submerged cultures of *A. blazei* inhibits α-glucosidase activity in vitro and exhibits hypoglycemic action in streptozotocin (STZ)-induced diabetic Sprague−Dawley (SD) rats. However, the molecule responsible for the hypoglycemic response, free of β-glucans and glycoproteins, is not known.

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Published on Web 03/02/2010

In the present study, we investigated the hypoglycemic actions of semipurified fractions from a submerged culture broth of *A. blazei* in STZ-diabetic SD rats and isolated the responsible chemical compounds.

MATERIALS AND METHODS

Materials. Streptozotocin (STZ), α-glucosidase, *p*-nitrophenyl-α-D-glucopyranoside, Acarbose, metformin, genistin, genistein, daidzin, daidzein, and thiobarbituric acid (TBA) were purchased from Sigma-Aldrich (St. Louis, MO). High-pressure liquid chromatography (HPLC) grade methanol, hexane, chloroform, ethyl acetate (EA), butanol, and acetic acid were obtained from J. T. Baker (Phillipsburg. NJ). *A. blazei* Murill, certified by Korea Seed & Variety Service (Whaseong, Republic of Korea), was generously donated by HK Biotech (Jinju, Republic of Korea). All other chemicals were of analytical grade.

Culture of *A blazei*. The liquid culture medium was composed of 10% enzyme-hydrolyzed soy flakes (kindly provided by HK Biotech), 1% proline, 1% xylose, 2% brown sugar, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.05% KH_2PO_4. After autoclaving at 121 °C for 30 min, we used the medium for the submerged-liquid culture of *A. blazei*. After activation of *A. blazei* on potato dextrose agar (PDA) plates by incubating at 25 °C until 80% confluence, we used one-quarter of the PDA plate as a subculture inoculum for the submerged-liquid culture in a 500 mL Erlenmeyer flask containing 300 mL of medium incubated for 7 days on a shaking incubator (120 rpm, 25 °C). The subculture broth was used as a main culture inoculum for the submerged-liquid culture of *A. blazei* in a 5 L fermenter jar (Kobiotech, Inchen, Republic of Korea) incubated for 3 days (120 rpm, 25 °C, 1% v/v/min) to obtain the *A. blazei* culture broth.

Preparation of the Extract and Fractions. The culture broth (3 L) was heated at 121 °C for 10 min, followed by filtering through a filter press coated with diatozoearth. The filtrate was centrifuged for 10 min at 5,000 rpm to remove particulate substances and then concentrated and freeze-dried. The dried material (50 g) dissolved in 150 mL of distilled water was added to 800 mL of 95% ethanol to obtain an 80% ethanol solution, which was maintained at refrigerator temperature overnight. After centrifugation at 10,000 rpm for 10 min at 4 °C, the supernatant was separated from the precipitate (10.4 g as a dried matter). The supernatant was concentrated to syrup (designated as a crude extract) and sequentially partitioned by hexane, chloroform, EA, and butanol to obtain, respectively, hexane fraction (HEF), chloroform fraction (CLF), EA fraction (EAF), and butanol fraction (BUF) and others. All fractions, including crude extracts, were freeze-dried. The portion of HEF, CLF, EAF, BUF, and others obtained from 50 g of freeze-dried material was found to be 0.6, 7.9, 12.1, 14.2, and 4.8 g, respectively.

α-Glucosidase Activity Assay. α-Glucosidase activity of samples was measured according to the method described previously (*19*). Briefly, α-glucosidase solutions (50 μL, 0.7 U) were added to wells of a 96-well plate containing an Acarbose solution (5 mg/50 μL distilled water) or sample solution (5 mg/50 μL distilled water), and absorbance at 405 nm was measured at zero time using an Anthos 2020 microplate reader (Cambridge, England). Five minutes later, a 50 μL substrate solution (5 mM *p*-nitrophenyl-α-D-glucopyranoside in 0.1 M phosphate buffer, pH 7.0) was added to the well containing α-glucosidase and sample or Acarbose and carefully mixed in room temperature. Five minutes later, after adding the substrate, absorbance at 405 nm was measured to calculate α-glucosidase activity.

Animal Experiments. *Induction of DM in SD Rats.* Male SD rats (5-weeks-of-age, 200−250 g) obtained from Samtako (Osan, Republic of Korea) were subjected to a standard pellet diet from Samtako in a temperature (23 ± 2 °C)-and humidity (50 ± 10%)-controlled facility with a 12 h light-and-dark cycle control system. One week later, the overnight-fasted SD rats were i.p. injected with 1 mL of STZ (60 mg/kg body weight, BW). STZ solution was freshly prepared in 0.01 M citrate buffer (pH 4.5) and maintained on ice prior to use. The fasting blood glucose level of rats was measured 72 h after STZ injection. Rats with >350 mg/dL glucose were considered as diabetic rats and used in the experiments 1 week after STZ injection (*20*). Animals had free access to food and water before and after the STZ injection. Rat care and experimental procedures were in accordance with Gyeongsang National University Animal Ethics Guidelines (GNU-LA-16).

Oral Glucose Tolerance Test (*OGTT*). Diabetic rats were fasted for 16 h and subjected to one of the following treatments (n = 6 per treatment group): control (200 mg water/kg BW), CLF (200 mg/kg BW), HEF (200 mg/kg BW), EAF (200 mg/kg BW), BUF (200 mg/kg BW), and metformin (500 mg/kg BW). Rats were administered p.o. with samples or metformin dissolved in 0.1 mL of distilled water. Thirty minutes later, glucose (3 g/kg BW) dissolved in 5 mL of distilled water was administered p.o. to each rat. Blood samples were collected in heparin-treated culture tubes from the tail vein by tail milking at 0, 30, 60, 120, and 180 min after glucose administration.

Fourteen Day Administration of EAF. Diabetic rats were divided into five groups (n = 9 per group): control (200 mg water/kg BW), EAF 100 (100 mg/kg BW), EAF 200 (200 mg/kg BW), EAF 400 (400 mg/kg BW), and metformin (500 mg/kg BW). Sample dissolved in 0.1 mL of distilled water was administered p.o. daily for 2 weeks. Body weight, food intake, and water intake were measured every day. On days 0, 7, and 15, after a 16 h fast, three rats per group were decapitated, and blood was collected in heparin-treated culture tubes for analyses of fasting glucose, insulin, and some other biochemical markers. Liver, kidney, and abdominal fat tissue were isolated, weighed, and stored at −21 °C for analysis of TBA reactive substances (TBARS) or glucose transport-4 (GLUT 4) protein.

Assay of Glucose and Insulin Levels. The contents of glucose and insulin in rat plasma were measured using a glucose assay kit (Sigma-Aldrich) and a rat insulin enzyme-linked immunosorbent assay kit (Mercodia, Uppsala, Sweden) according to the standard procedures provided by the companies. The reaction was stopped by adding 12 N H_2SO_4 for the glucose assay and 0.5 M H_2SO_4 for the insulin assay to give a colorimetric end point. Absorption at 540 nm for glucose and 450 nm for insulin was measured on a Anthos 2020 microplate autoreader.

Assay of GLUT-4 Protein and TBARS. Fat tissues were homogenized in test tubes containing protein extraction buffer (0.25 M sucrose in 10 mM HEPES, pH 7.5) and were placed in an ice bath with a homogenizer (Wheaton Science Products, Milliville, PA), followed by filtration with a 0.45 μm sterile filter (Sartorius Biotech GmbH., Goettingen, Germany). Filtrates were used for the GLUT-4 assay as described previously (*21*). Liver and kidney samples were minced and homogenized with a homogenizer in ice-cold 1.15% KCl to make a 25% homogenate. TBARS value was determined as previously described (*22*). Briefly, to 0.1 mL of the homogenate, 0.2 mL of 8.1% sodium dodecyl sulfate (SDS), 1.5 mL of 1% phosphoric acid, 0.2 mL of distilled water, and 1 mL of 0.6%TBA were added and then the mixture was heated in a boiling water bath. Forty-five minutes after heating, the reaction mixture was cooled in an ice bath and extracted with 4 mL of *n*-butanol. After centrifugation at 1,000*g* for 5 min, the absorbance of the *n*-butanol layer was determined at 532 nm using a model DU650 spectrophotometer (Beckman, Fullerton, CA). Values are expressed as nmol malonedialdehyde (MDA)/g wet tissue. 1,1,3,3-Tetraethoxypropane was used as a standard for malondialdehyde.

Glutamate−Oxalate Transaminase (GOT) and Glutamate−Pyruvate Transaminase (GPT) Assay. The activity of GOT and GPT in plasma samples was measured by GOT and GPT assay kits (Asan Pharmaceutical, Seoul, Republic of Korea) according to the standard procedure provided by the company. Plasma samples (0.2 mL) were used, and the absorbance developed for 60 min (GOT) and 30 min (GPT) was determined at 505 nm using a model DU 650 spectrophotometer.

Cholesterol and Triglyceride in Plasma. Triglyceride and total cholesterol contents in plasma were determined by an EnzyChrom cholesterol assay kit (BioAssay Systems, Hayward, CA) and an EnzyChrom triglyceride assay kit (BioAssay Systems) according to the standard procedures provided by the company. The absorbance was measured by a model DU 650 spectrophotometer.

Hemoglobin A1c (HbAlc) Assay in Blood. HbAlc was determined by an HbA1c micro column assay (Bio-Rad, Hercules, CA) according to the procedure provided by Kosova and Kara (*23*). Briefly, each blood sample was mixed with lysis solution composed of 0.33% polyethoxyethanol and 0.6 M borate in a 2-*N*-morpholino ethanesulfonic acid buffer. HbA1c was separated from other types of Hb using a Bio-Rad HbA1c micro column packed with a weak cation exchange resin. HbAlc was measured by detecting the color developed at 550 nm by a model DU 650 spectrophotometer. Data were expressed as percentage of HbA1c against total Hb.

Analysis of Isoflavonoids. Isoflavonoids (genistin, genistein, daidzin, and daidzein) in samples were analyzed as previously described (*24*) with slight modifications. Briefly, the sample (100 mg) was hydrolyzed by heating in a boiling water bath with 15 mL of 6 N HCl for 12 h, followed by neutralization with 6 N NaOH, and filtering with filter paper. The resultant was rotoerevaporated under vacuum (EYELA, Tokyo, Japan), dissolved in 20 mL of methanol, and sonicated three times for 5 min using a Sonorex Super RK 514 BH ultrasonicator (Bandelin, Berlin, Germany). After concentration, the total volume was adjusted to 5 mL with methanol, followed by filtration with a 0.20 μm Sartorius filter. Following further concentration to 200 μL, the sample was used for HPLC analysis, utilizing a model M930 HPLC apparatus (Young-Lin, Seoul, Republic of Korea) equipped with a 3.9 × 300 mm μ-Bondapak C18 column (Waters, Milford, MA). The injection volume was 20 μL. The sample was eluted with a mobile phase of water/methanol/acetic acid (75/25/0.1, v/v) at a flow rate of 1 mL/min and was examined at 254 nm.

Statistical Analysis. The results are presented as the mean ± SD. Body weight, food intake, and water intakes were compared by two-way ANOVA. All other data were analyzed by Duncan's multiple range tests or *t*-tests.

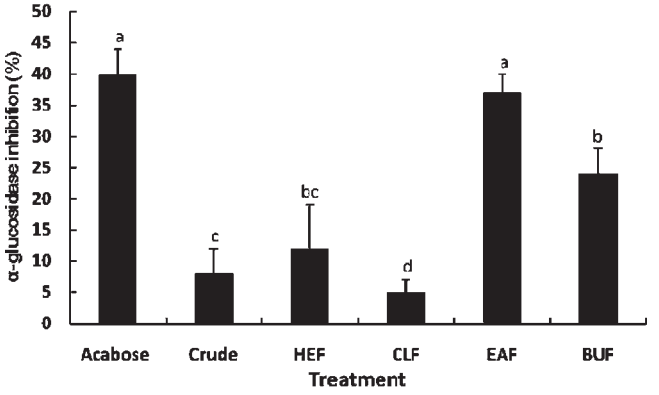

Figure 1. Inhibition of α-glucosidase activity by semipurified fractions from hot-water extracts of the submerged-culture broth of *A. blazei* Murill. For the assays, 5 mg each of Acarbose, crude extract, HEF, CLF, EAF, and BUF were used. The results represent the mean ± SD (n = 3). Means of treatments with different lowercase letters represent significant difference at $p < 0.05$ by Duncan's multiple range tests.

RESULTS

Inhibition of α-Glucosidase Activity by Semipurified Fractions. Prior to determining the hypoglycemic effects of semipurified fractions from hot-water extracts of the submerged culture broth of *A. blazei*, the inhibitory effects of the semipurified EAF, CLF, HEF, and BUF were determined on α-glucosidase activity (**Figure 1**). EAF inhibited activity by 37%, which was similar to that resulting from Acarbose (5 mg), a positive compound for α-glucosidase inhibition, whereas BUF showed 23% inhibition, and other fractions were even less effective. We next determined whether the various fractions could decrease blood glucose levels in STZ-induced diabetic SD rats.

Hypoglycemic Effects of Semipurified Fractions in STZ-Induced Diabetic Rats. In OGTT (**Figure 2**), EAF (200 mg/kg BW) showed a significant hypoglycemic effect within 30 min after oral glucose administration to diabetic rats. EAF significantly ($P < 0.01$) reduced the blood glucose level at 60, 120, and 180 min compared with that of the control. The efficacy of EAF was similar to that of metformin. BUF (200 mg/kg BW), which strongly inhibited α-glucosidase activity as shown in **Figure 1**, showed no significant effect at 30 min but showed a significant effect ($P < 0.01$) at 180 min after oral glucose administration, relative to that of control treatment. Blood glucose reducing effect of CLF was similar to that of BUF, but HEF was not effective on the blood glucose level at 180 min. These results implicated EAF as the semipurified fraction most worthy of further study in diabetic rats.

Prolonged experiments on STZ-induced diabetic rats were performed to elucidate the hypoglycemic effects of EAF. EAF (100, 200, and 400 mg/kg BW) treated over 14 days in STZ-induced diabetic rats significantly ($P < 0.05$) increased BW but significantly ($P < 0.05$) reduced food intake and water consumption relative to those of control rats and similar to those of metformin (**Table 1**). The reduction of food intake by EAF treatment may have been partly due to the effects of some nutrients contained in EAF. EAF decreased blood glucose levels for 14 days in a dose-dependent manner (**Figure 3**, left). EAF (> 200 mg/kg BW) significantly ($P < 0.05$) reduced blood glucose levels at 7 days as compared to that in the control treatment and further reduced blood glucose ($P < 0.01$) at

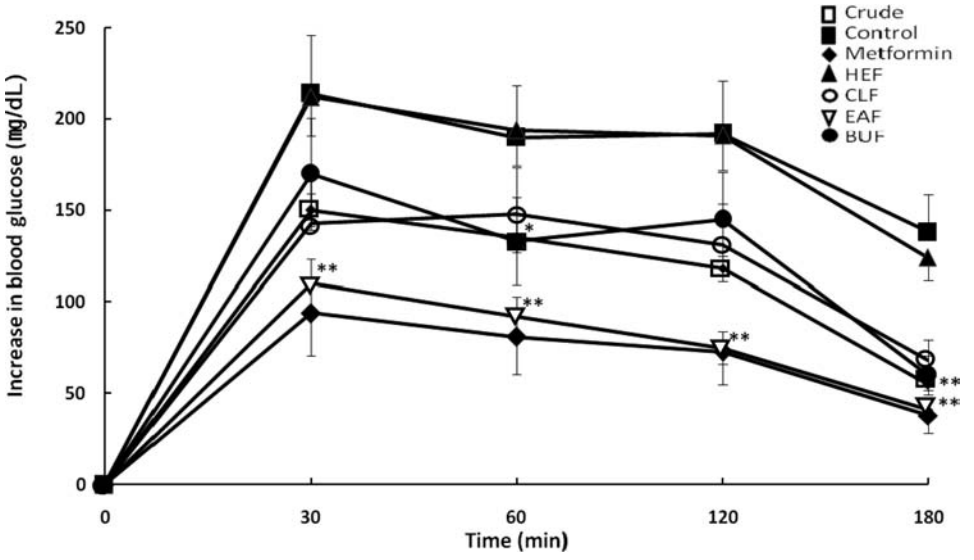

Figure 2. Increase in blood glucose levels of STZ-induced diabetic SD rats administered semipurified fractions of submerged-cultured broth of *A. blazei* Murill. Diabetic rats were administered control (0.2 mL of distilled water), crude extract (200 mg/kg BW), HEF (200 mg/kg BW), CLF (200 mg/kg BW), EAF (200 mg/kg BW), BUF (200 mg/kg BW), and metformin (500 mg/kg BW). Mean with asterisk (* and **) represents significant ($P < 0.05$ and $P < 0.01$, respectively) difference from control mean at given time by *t*-tests.

Table 1. Body Weight, Water Intake, and Food Intake of STZ-Diabetic SD Rats Treated with EAF

	body weight[b] (g/rat)		water intake[c] (mL/rat/day)		food intake[c] (g/rat/day)	
treatment[a]	before	after	before	after	before	after
control	227 ± 15[d] a	230 ± 10 c	161 ± 10 a	91 ± 11 a	21.9 ± 4 a	24.7 ± 3 a
EAF 100	242 ± 17 a	278 ± 30 ab	160 ± 11 a	58 ± 10 b	19.4 ± 4 a	17.8 ± 2 b
EAF 200	269 ± 21 a	284 ± 13 ab	155 ± 14 a	78 ± 10 b	20.5 ± 3 a	15.9 ± 2 b
EAF 400	263 ± 10 a	258 ± 13 b	165 ± 15 a	73 ± 14 b	18.6 ± 4 a	14.0 ± 3 b
Metformin 500	249 ± 23 a	311 ± 24 a	162 ± 12 a	71 ± 12 b	18.4 ± 3 a	16.2 ± 2 b

[a] SD rats were orally administered daily for 2 weeks with control (200 μL distilled water/kg BW), EAF 100 (100 mg/kg BW), EAF 200 (200 mg/kg BW), EAF 400 (400 mg/kg BW), and metformin (500 mg/kg BW). [b] Body weight was measured just before sample treatment and at day 14. [c] Both water and food intake was measured each day for 2 days before sample treatment and thereafter measured daily for 14 days. [d] Mean ± SD for body weight and mean ± SE for water and food intake. Means with different lowercase letters in the same column represent significant difference at $p < 0.05$ by Duncan's multiple range tests.

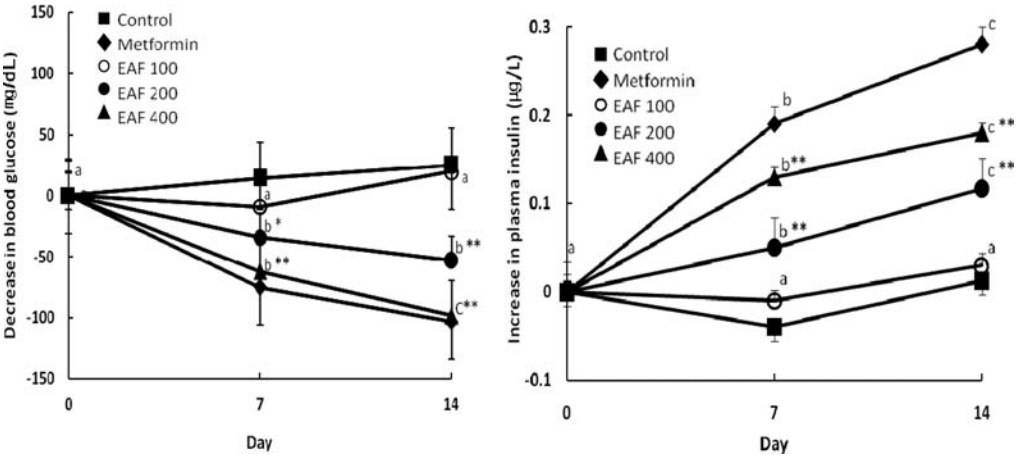

Figure 3. Decrease in blood glucose and increase in plasma insulin levels in STZ-induced diabetic SD rats administered EAF. Each day, rats received the control (0.2 mL of distilled water/kg BW), EAF 100 (100 mg/kg BW), EAF 200 (200 mg/kg BW), EAF 400 (400 mg/kg BW), and metformin (500 mg/kg BW). Blood was collected in heparin-treated culture tubes from rat tails 16 h after fasting. The average glucose levels in blood at day zero of control, EAF 100, EAF 200, EAF 400, and metformin treatments were 504, 476, 595, 513, and 533 mg/dL, respectively. The average insulin levels in plasma at day zero of the control, EAF 100, EAF 200, EAF 400, and metformin treatments were 0.50, 0.55, 0.50, 0.52, and 0.45 μg/L, respectively. Means with different lowercase letters given in the same line represent significant difference at $p < 0.05$ by Duncan's multiple range tests. Mean with asterisk (* and **) represents significant ($P < 0.05$ and $P < 0.01$, respectively) difference from the control mean at the given treatment time by *t*-tests.

14 days when compared with that in the control. The efficacy of EAF (400 mg/kg BW) was closely similar to that of metformin, but 100 mg EAF/kg BW was not sufficient to produce a hypoglycemic response.

Effects of EAF on Hypoglycemic Biomarkers in STZ-Induced Diabetic Rats. To elucidate the molecular mechanism(s) of EAF on the reduction of blood glucose levels in STZ-induced diabetic rats in the prolonged (14 day) experiments, we investigated biochemical markers including insulin level in plasma and GLUT-4 expression in adipose tissue, and others related to increased or decreased blood glucose. **Figure 3**, right depicts the increase in plasma insulin in STZ-induced diabetic rats treated with EAF for 14 days. Using 200 mg/kg BW EAF, the plasma insulin level was significantly higher on day 7, compared to that of both the control ($P < 0.05$) and the day 0 level ($P < 0.05$), and the effect was greater on day 14. EAF at 400 mg/kg BW also significantly ($P < 0.01$) increased the insulin level on days 7 and 14, relative to that of the control, and the effect was slightly lower than that of metformin. However, treatment with 100 mg/kg BW EAF was not sufficient to increase plasma insulin levels throughout the experimental period.

Expression of GLUT-4 protein was determined in adipose tissues from STZ-rats treated with EAF (100, 200, and 400 mg/kg BW) on day 14 (**Figure 4**). The expression of GLUT-4 protein was linearly increased in an EAF dose-dependent manner. The expression in the presence of 400 mg/kg BW EAF was

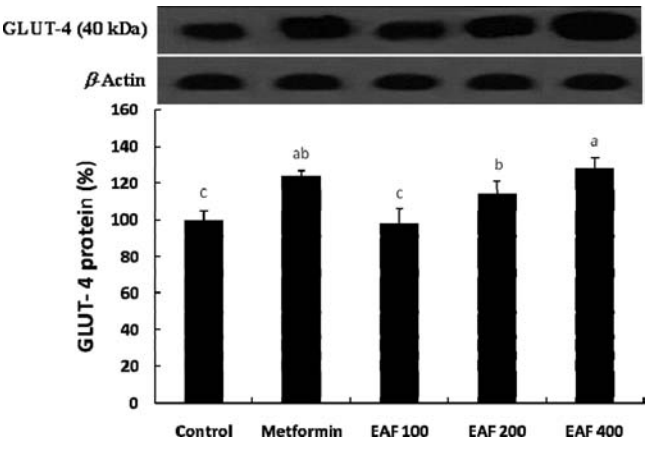

Figure 4. Expression of GLUT-4 protein in the fat tissues of STZ-diabetic SD rats administered EAF. Rats were administered once a day with control (200 μL of distilled water/kg BW), EAF 100 (100 mg/kg BW), EAF 200 (200 mg/kg BW), EAF 400 (400 mg/kg BW), and metformin (500 mg/kg BW). Abdominal fat samples from rats were collected on day 15 post-sample treatment. Means of treatment groups with different lowercase letters represent significant difference at $p < 0.05$ by Duncan's multiple range tests.

significantly ($P < 0.05$) different from that of control treatment and closely resembled the effect produced by metformin, consistent

Table 2. Levels of Some Biochemical Markers in Blood from STZ-Diabetic SD Rats Treated with EAF

treatment[a]	triglyceride (mg/dL)	cholesterol (mg/dL)	GOT (U/L)	GPT (U/L)	HbA1c (%)
control	410 ± 21[b] a	142 ± 15 a	164 ± 14 a	164 ± 19 a	75 ± 8 a
EAF 100	366 ± 32 b	122 ± 15 ab	154 ± 20 a	163 ± 20 a	71 ± 5 a
EAF 200	210 ± 41 c	103 ± 23 bc	146 ± 10 a	149 ± 12 a	68 ± 8 a
EAF 400	156 ± 37 d	96 ± 8 c	112 ± 17 b	128 ± 15 b	65 ± 3 a
metformin 500	275 ± 41 c	93 ± 8 c	107 ± 19 b	100 ± 8 b	73 ± 6 a

[a] Rats were orally administered daily for 2 weeks with control (200 μL distilled water/kg BW), EAF 100 (100 mg/kg BW), EAF 200 (200 mg/kg BW), EAF 400 (400 mg/kg BW), and metformin (500 mg/kg BW). Blood samples were collected in heparin-treated culture tubes from tail veins on day 14 post-sample treatment. [b] Mean ± SD of three independent experiments. Means with different lowercase letters in the same column represent significant difference at $p < 0.05$ by Duncan's multiple range tests.

Table 3. TBARS in Liver and Kidney from STZ-Diabetic SD Rats Treated with EAF

treatment[a]	liver (nmol/g)	kidney (nmol/g)
control	31.7 ± 0.9[b] a	42.0 ± 1.6 a
EAF 100	32.4 ± 2.2 a	40.5 ± 1.3 a
EAF 200	28.3 ± 2.5 a	39.7 ± 1.8 a
EAF 400	21.4 ± 2.1 b	37.1 ± 1.0 b
metformin 500	21.3 ± 1.1 b	39.5 ± 1.5 a

[a] Rats were orally administered daily for 2 weeks with control (200 μL distilled water/kg BW), EAF 100 (100 mg/kg BW), EAF 200 (200 mg/kg BW), EAF 400 (400 mg/kg BW), and metformin (500 mg/kg BW). Fat samples were collected in heparin-treated culture tubes from rats on day 14 post-sample treatment. [b] Mean ± SD ($n = 3$). Means with different lowercase letters in the same column represent significant difference at $p < 0.05$ by Duncan's multiple range tests.

Table 4. Contents of Major Isoflavonoids in Semi-Purified Fractions from Hot Water Extracts of the Submerged-Culture Broth of *A. blazei* Murill

	isoflavonoid[b] (μg/g)			
fraction[a]	genistein	genistin	daidzein	daidzin
crude extract	654 ± 24[c] b	580 ± 12 c	700 ± 23 b	714 ± 30 b
HEF	2 ± 1 c	27 ± 2 d	11 ± 1 d	–[d]
CLF	598 ± 21 b	806 ± 21 b	606 ± 30 c	733 ± 29 b
EAF	993 ± 23 a	946 ± 32 a	1,047 ± 35 a	813 ± 30 a
BUF	–	–	–	–

[a] Crude extract was fractionated into HEF, CLF, EAF, and BUF. [b] Isoflavonoids were analyzed by a reversed-phase HPLC using a C18 column and monitored at 258 nm. [c] Mean ± SD ($n = 3$). Means with different lowercase letters in the same column represent significant difference at $p < 0.05$ by Duncan's multiple range tests. [d] Not detectable.

with the suggestion that EAF is sufficient to induce GLUT-4 to regulate blood glucose levels in diabetes.

Table 2 summarizes the triglyceride, cholesterol, GOT, GPT, and HbA1c levels in blood from STZ-induced diabetic rats treated with EAF (100, 200, and 400 mg/kg BW) on day 14. EAF significantly ($P < 0.05$) reduced triglyceride and cholesterol levels in a dose-dependent manner. The efficacy of 400 mg EAF/kg BW was greater ($P < 0.05$) in triglyceride reduction than that of metformin and was similar to metformin with respect to cholesterol reduction. Similar results were obtained in GPT and GOT levels. Meanwhile, the HbA1c level was not affected by any EAF treatment. Such effects were seen in TBARS contents in liver and kidney tissues (**Table 3**). Only 400 mg EAF/kg BW significantly ($P < 0.05$) suppressed the formation of TBARS in both tissues, relative to that in the control. The contents of TBARS in control liver and kidney were 31 and 42 nmol/g tissues, respectively, but these values were significantly ($P < 0.05$) reduced to 21 and 37 nmol/g tissues, respectively, by treatment with 400 mg EAF/kg BW. The effect of this EAF concentration was similar to that of metformin.

Contents of Isoflavonoids in Semipurified Fractions. The contents of the major isoflavonoids (genistein, genistin, daidzein, and daidzin) in the semipurified EAF, HEF, CLF, and BUF were determined by HPLC. The results are shown in **Table 4**. All semipurified fractions except for BUF contained substantial amounts of the four isoflavonoids. EAF contained the highest amount of genistein (993 μg/g), genistin (946 μg/g), daidzein (1,047 μg/g), and daidzin (813 μg/g), whereas HEF contained the least amounts of all four compounds, ranging from 27 μg/g of genistin to the absence of daidzein. These results correlated with the hypoglycemic activity of semipurified fractions (**Figures 2** and **3**), indicating that these isoflavonoids could party contribute to the hypoglycemic actions of EAF and the hot-water extract of the submerged-culture broth of *A. blazei* in STZ-induced diabetic rats.

DISCUSSION

The present study clearly revealed an EAF-mediated hypoglycemic action in STZ-diabetic rats over a 2 week period. The efficacy of EAF was equal to that of metformin, a drug commonly used to treat diabetes patients. These results suggest that EAF can potentially control glucose metabolism in humans since the STZ-induced diabetic rat, in which the irreversibly destroyed β-islet cells of the pancreas cause marked depletion (but not the absence) of insulin, is an animal model of human insulin-dependent DM (IDDM) (*20*, *25*).

In STZ-induced diabetic rats, EAF presently exhibited a hypoglycemic action by increasing plasma insulin level and GLUT-4 expression in fat tissues (**Figures 3** and **4**). Consequently, EAF suppressed the contents of triglyceride, cholesterol, GOT, and GPT in blood, and TBARS contents in liver and kidney tissues. Hence, EAF could act as either an insulinotropic agent, inducing the intact functional β-cells to produce insulin, or protect the functional β-cells from further deterioration so that they remained active and capable of insulin production (*25*). Although insulin has become one of the most important therapeutic agents known to medicine so far, there is a continuing effort to find insulin substitutes from mushroom and plant sources for the treatment of diabetes. Some fruit bodies of mushrooms and the submerged-culture broths of mushroom mycelia have been shown to possess insulin-releasing action both in vitro and in vivo (*5*, *26*, *27*). Similar effects were observed in plant extracts of *Lagerstroemia speciosa* (*4*), *Medicago sativa* (*28*), and *Acanthopanax senticosus* (*3*). The principle compounds have been identified as β-glucans and glycoproteins from mushrooms, and as flavonoids from plants.

Presently, EAF reduced TBARS formation in the liver and kidney in STZ-induced diabetic rats (**Table 3**), which could be, in part, due to its hyperinsulinemic and hypoglycemic effects (**Figure 3**). Hypoinsulinemia in diabetes increases lipid peroxidation through enhanced fatty acyl CoA oxidase activity, which initiates β-oxidation of fatty acids (*29*, *30*). Hyperglycemia in STZ-treated rats produces hydrogen peroxide (*20*, *31*), which is degraded to superoxide and the hydroxyl radical, which causes peroxidation of lipids and a subsequent increase in TBARS. Hence, EAF could protect from the development of chronic

complications of diabetes by decreasing the alteration of membrane fluidity caused by lipid peroxidation.

The potential hypoglycemic components in EAF were isolated from EAF by HPLC and were found to be isoflavonoids, such as genistein, genistin, daidzein and daidzin, ranging from 1,047 μg/g for daidzein to 813 μg/g for diadzin (**Table 4**). The isoflavonoids in EAF can be derived from the culture media of soybean flakes containing isoflavonoids, mainly genistein, genistin, daidzein, and daidzin (*32*). Some flavonoids isolated from plant sources and soy products containing isoflavonoids are used for the treatment of DM (*3*, *4*, *28*, *33*−*37*). It is also well known that genistein inhibits the activity of α-glucosidase in vitro (*32*). Soluble β-glucan from mushrooms, mushroom mycelia, and the culture broth of mushroom mycelia have potent hypoglycemic action in chemical-induced diabetic rats through the inhibition of intestinal α-glucosidase and enhancement of insulin secretion (*7*, *25*). However, presently the effect of β-glucan was ruled out because of the absence of β-glucan in EAF, which was removed by treatment with 80% ethanol. Hence, isoflavonoids contained in the submerged-culture broth of *A. blazei* might contribute to reduced blood glucose levels in STZ-induced diabetic rats. The submerged-culture broth of *A. blazei*, which contains isoflavonoids, could be useful in the treatment of diabetes because the STZ-induced diabetic rat used presently is a relevant model of human IDDM.

In conclusion, EAF has hypoglycemic potential in STZ-diabetic rats. The hypoglycemic action might be attributed to isoflavonoids including genistein, genistin, daidzein, and daidzin and other substances in EAF. Additional pharmacological studies are being carried out to elucidate its mechanism of action as well as to assess the use of EAF for the treatment of human DM.

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Received for review October 20, 2009. Revised manuscript received January 3, 2010. Accepted February 15, 2010. This study was partly supported by a scholarship from the BK21 Program and a grant from Korea Research Foundation, the Ministry of Education, Science and Technology, Republic of Korea.