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Hypoglycemic Effect of Fermented Soymilk Extract in STZ-induced Diabetic Mice

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Abstract

This study investigated the hypoglycemic effect of fermented soymilk extract (FSE) in STZ-induced diabetic mice. FSE was prepared via fermentation of soymilk with *Bacillus subtilis* followed by methanol extraction. The hypoglycemic effect was determined by inhibitory activities against α-glucosidase and α-amylase as well as the alleviation of postprandial glucose level. The non-fermented soymilk extract (SE) was used as control in this experiment. FSE showed higher ($p < 0.05$) inhibitory activities than SE against α-glucosidase and α-amylase. The IC_{50} values of FSE for α-glucosidase and α-amylase were 0.77 and 0.94 mg/mL, respectively, which were comparable or even superior to those of acarbose (0.79 and 0.68 mg/mL, respectively). In addition, a further suppression on the postprandial blood glucose levels were observed in the FSE than SE group for both STZ-induced diabetic mice and normal mice. Furthermore, FSE significantly lowered the incremental area under the curve (AUC) in the diabetic mice and the AUC in normal mice corroborated the hypoglycemic effect of FSE ($p < 0.05$). Results from this study suggest that FSE may help decrease the postprandial blood glucose level via inhibiting α-glucosidase and α-amylase and the usefulness of FSE was proven to be better than SE.

Key words: fermented soymilk extract (FSE), α-glucosidase, α-amylase, postprandial hyperglycemia, STZ-induced diabetes

INTRODUCTION

Diabetic mellitus is the most serious, chronic metabolic disorder and is characterized by high blood glucose levels (1,2). The prevalence of diabetes mellitus is increasing markedly because of an aging population, increased urbanization, and more sedentary lifestyles. Keeping blood glucose level close to normal and preventing diabetic complications are the major goals in the treatment of diabetic mellitus (3,4). Optimizing both fasting blood glucose and postprandial glucose level is important in achieving normal glucose level (5). It was reported that postprandial glucose levels could be a better marker of glycemic control than fasting blood glucose levels in patients with type 2 diabetes (6). The control of postprandial hyperglycemia is critical in the early therapy for diabetes (7,8). Controlling postprandial glucose levels is an also important strategy in the prevention of type 2 diabetes (9).

One therapeutic approach to decrease postprandial hyperglycemia is to retard absorption of glucose through inhibition of carbohydrate-hydrolyzing enzymes, e.g., α-amylase and α-glucosidase, in the digestive organs (10-13). Clinical studies have documented that α-glucosidase inhibitor is effective in controlling both fasting and postprandial hyperglycemia in patients with diabetes (10,14,15), and the relative risk of type 2 diabetes could be decreased by α-glucosidase inhibitors in subjects with impaired glucose tolerance and obesity (9). Antidiabetic agents such as acarbose, voglibose, miglitol that inhibit α-glucodsidase and α-amylase are widely used in the treatment of patients with type 2 diabetes (16,17). However, chronic use of three agents could result in side effect such as flatulence, abdominal cramping, vomiting and diarrhea so that their use may be limited (18). Therefore, numerous studies have been carried out to find α-amylase and α-glucosidase inhibitors from natural products in an attempt to reduce any possible side effects of antidiabetic medicines. In addition, several α-glucosidases have been recently screened and developed from natural sources (19-22).

Soybean, the most important legume in Asian diet, is rich in high-quality protein because it is rich in lysine and other essential amino acids (23). In addition to proteins, they contain various nutritious and functional components such as isoflavones. The interest in the potential health effects of soybean and soy isoflavones is growing as epidemiological studies have associated a diet rich

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in isoflavones with a lower risk of certain diseases (24-26). Recently, soybean and soy protein have received much attention for their preventive effects on chronic disease (27-29). However, two of the main oligosaccharides in soybean, raffinose and stachyose, are not nutritionally useful because these are fermented by microbes present in the gut, the results are flatulence and discomfort. Fermentation of soymilk by mixed cultures of bifidobacteria and lactic acid bacteria has been shown to effectively decrease the content of these two non-digestable oligosaccharides. Fermentation has been known as chemical reaction that splits complex organic compounds into relatively simple substances. Fermentation of legumes has been reported to cause a improvement in the nutritional value, increasing proteins digestibility, monosaccharide content, vitamin B family biosynthesis and to decrease non-nutritive factors (30). Soybean products fermented with *Bacillus subtilis* are widely consumed in Asia, including Chungkookjang and natto.

B. subtilis, which is safe and grow rapidly and easy to be scale-up for mass culture, has been the good organism to develop a probiotic diet adjunct. Kuo et al. (31) reported that *Bacillus subtilis*-fermented natto hydrolyzed daidzin and genistin to daidzein and genistein, respectively, in black soymilk. Soymilk is the water extract of soybeans. Soymilk is a colloidal dispersion extracted from ground soybeans; therefore, most components that are present in the seed are present in soymilk. During fermentation, the active compounds in soymilk will be exposed and these may have effectiveness such as antidiabetic activity. Therefore, this study was designed to examine the effect of FSE, which was made of soymilk fermented with *Bacillus subtilis,* on blood glucose levels in normal and streptozotocin (STZ)-induced diabetic mice. In the present study, we report the nutritional benefit of FSE by showing the antidiabetic effect of FSE via the inhibition against α-glucosidase and α-amylase as well as the suppression of postprandial hyperglycemia.

MATERIALS AND METHODS

Preparation of FSE

Soymilk was purchased from Donghwa food, Inc. (Yangsan, Korea). Soymilk was fermented by *Bacillus subtilis* isolated from Chungkookjang for 6 hr at 40°C under aerobic conditions. Non-fermented as well as the fermented soymilk were freeze-dried, powdered and extracted with ten volumes of 100% methanol for 12 hr three times at room temperature. The filtration of the extracted solution and evaporation under reduced pressure yielded methanol extract. After the extract was thoroughly dried for complete removal of solvent, the dried extract was then stored in a deep freezer (-80°C).

Alpha-glucosidase inhibitory assay *in vitro*

The α-glucosidase inhibitory assay was done by the chromogenic method developed by Watanabe et al. (32) using a readily available yeast enzyme. Briefly, yeast α-glucosidase (0.7 U, Sigma) was dissolved in 100 mM phosphate buffer (pH 7.0) containing 2 g/L bovine serum albumin and 0.2 g/L NaN_3 and used as an enzyme solution. 5 mM *p*-nitrophenyl-α-D-glucopyranoside in the same buffer (pH 7.0) was used as a substrate solution. The 50 μL of enzyme solution and 10 μL of sample dissolved in dimethylsulfoxide at the 5 mg/mL concentration were mixed in a well, and absorbance at 405 nm was measured using a microplate reader. After incubation for 5 min, substrate solution (50 μL) was added and incubated for another 5 min at room temperature. The increase in absorbance from zero time was measured. Inhibitory activity was expressed as 100 minus relative absorbance difference (%) of test compounds to absorbance change of the control where test solution was replaced by carrier solvent. The measurements were performed in triplicate and IC_{50} value, i.e., the concentration of the extract that results in 50% inhibition of maximal activity, was determined.

Alpha-amylase inhibitory assay *in vitro*

The α-amylase inhibitory activity was assayed in the same way as described for a α-glucosidase inhibitory assay except that porcine pancreatic amylase (100 U, Sigma) and blocked *p*-nitrophenyl-α-D-maltopentoglycoside (Sigma, St Louis, MO, USA) were used as enzyme and substrate, respectively.

Experimental animals and diabetes inducement

Four-week old male mice (ICR, Orient, Inc., Seoul, Korea) were kept under a 12 hr light/12 hr dark cycle with room temp. controlled. The animals were maintained with pelleted food, while tap water was *ad libitum*. After an adjustment period of 2 weeks, diabetes was induced by intraperitoneal injection of STZ (60 mg/kg i.p.) freshly dissolved in a citrate buffer (0.1 M, pH 4.5) for the fasted (18 hr) animals. After seven days, tail bleeds were performed and animals with a blood glucose concentration above 250 mg/dL were considered to be diabetic.

Measurement of postprandial blood glucose level

Both normal mice and STZ-induced diabetic mice fasted overnight were randomly divided into three groups. Fasted animals were deprived of food for at least 12 hr but allowed free access to water. After overnight fasting, the mice were administered orally either soluble starch (2 g/kg body weight) alone (control) or starch with

non-fermented soymilk extract (SE) or fermented soymilk extract (FSE) (500 mg/kg body weight). Blood samples were taken from the tail vein at 0, 30, 60 and 120 min. Blood glucose was measured using a glucometer (Roche Diagnostics GmbH, Germany) The blood glucose level was expressed in increments from the baseline. Incremental areas under the response curve (AUC) were calculated using the trapezoidal rule (33).

Statistical analysis

The data were represented as mean±SD. The statistical analysis was performed with SAS program (version 8.02). The values among groups were evaluated by one-way analysis of variance (ANOVA) followed by post-hoc Duncan's multiple range tests. Differences between FSE and acarbose were assessed using student t-test.

RESULTS AND DISCUSSION

Inhibitory effect of FSE on α-glucosidase and α-amylase *in vitro*

The inhibitory effect of FSE against yeast α-glucosidase is shown in Fig. 1. The FSE inhibited the α-glucosidase more effectively than SE ($p<0.05$). The SE inhibited α-glucosidase activity by 7.04, 13.53, 26.55, 37.48, and 43.76% at the concentration of 0.1, 0.25, 0.5, 0.75, and 1 mg/mL *in vitro*, respectively. The FSE inhibited the α-glucosidase activity by 14.2, 22.24, 41.42, 49.87, and 54.67% at concentrations of 0.1, 0.25, 0.5, 0.75, and 1 mg/mL *in vitro*, respectively. Acarbose, an α-glucosidase inhibitor, used as an oral hypoglycemic agent, inhibited the enzyme activity by 40.31% at concentration of 0.5 mg/mL. The α-glucosidase inhibitory activity of FSE at the concentration of 0.5 mg/mL was comparable to that of acarbose (0.5 mg/mL). The inhibitory effect of FSE against α-amylase is shown in Fig. 2. The FSE inhibited the α-amylase more effectively than SE ($p<0.05$). The SE inhibited α-amylase by 7.42, 13.39, 27.74, 32.90, and 44.19% at concentration of 0.1, 0.25, 0.5, 0.75, and 1 mg/mL *in vitro*, respectively. The FSE inhibited α-amylase by 12.58, 20.81, 30.32, 41.61, and 52.26% at concentration of 0.1, 0.25, 0.5, 0.75, and 1 mg/mL *in vitro*, respectively. The IC_{50} values of FSE for α-glucosidase and α-amylase were 0.77 and 0.94 mg/mL, respectively, which were comparable to those of acarbose (0.79 and 0.68 mg/mL, respectively) (Table 1).

α-Glucosidase is one of a number of glucosidases located in the brush-border surface membrane of intestinal cells, and is a key enzyme of carbohydrate digestion (34). Similarly, α-amylase catalyses the hydrolysis of α-1,4-glucosidic linkages of starch, glycogen and various oligosaccharides and α-glucosidase further breaks down the disaccharides into simpler sugars, readily available for the intestinal absorption. The inhibition of their activity, in the digestive tract of humans, is considered to

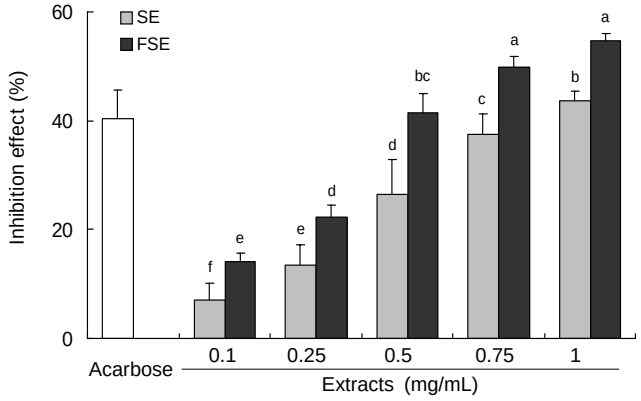

Fig. 1. Inhibitory activity of fermented soymilk extract on α-glucosidase. The final concentration of soymilk extract (SE) and fermented soymilk extract (FSE) were 0.1, 0.25, 0.5, 0.75, 1 mg/mL. Each value is expressed as mean±SD in triplicate experiments. [a-f]Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test. The concentration of acarbose was 0.5 mg/mL.

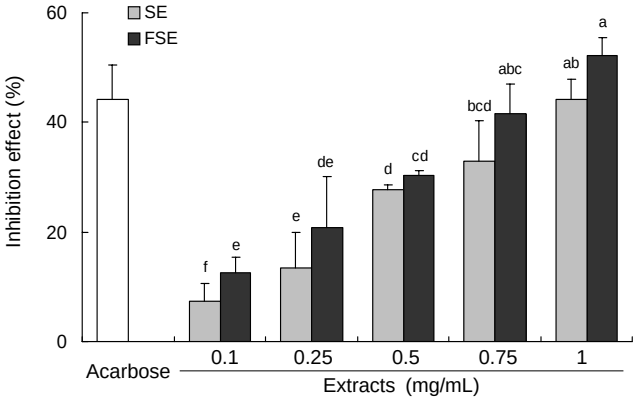

Fig. 2. Inhibitory activity of fermented soymilk extract on α-amylase. The final concentration of soymilk extract (SE) and fermented soymilk extract (FSE) were 0.1, 0.25, 0.5, 0.75, 1 mg/mL. Each value is expressed as mean±SD in triplicate experiments. [a-f]Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test. The concentration of acarbose was 0.5 mg/mL.

Table 1. IC_{50} value[1)] of inhibitory activity of fermented soymilk extract on α-glucosidase and α-amylase

Sample	IC_{50} (mg/mL)	
	α-glucosidase	α-amylase
Acarbose	0.79±0.03	0.68±0.07*
FSE	0.77±0.07	0.94±0.09

[1)]IC_{50} value is the concentration of sample required for 50% inhibition. Each value is expressed as mean±SD (n=3).
FSE: fermented soymilk extract.
*Significantly different from acarbose at $p<0.05$.

be effective to control diabetes by diminishing the absorption of glucose decomposed from starch by these enzymes (35,36).

Our data showed that FSE had higher inhibitory activities than SE on α-glucosidase and α-amylase, suggesting the fermentation of SE with *B. subtilis* was proven to be useful in terms of diabetic control. Fermentation consists of modifying food by microorganisms that grow and reproduce and consume part of the substrate and enrich it with the products of their metabolism. The results suggest that the amount of compounds inhibiting the enzymes was increased during fermentation of soymilk. Soymilk is the most important traditional soy foods made from whole soybean. The consumption of soymilk is increasing because of the high awareness of consumers of the health beneficial functions of soy foods (37). Soymilk contains beneficial components for human health, such as soy protein, peptides, isoflavones. The fermentation of soymilk was suspected to result in various compositional and functional changes as the fermentation of soybeans produce a large variety of peptides and amino acid by different kinds of microorganism. It has been documented that the inhibition of α-glucosidases and α-amylases resulted in a delayed carbohydrate digestion and glucose absorption with attenuation of postprandial hyperglycemia excursions (38). The inhibition of α-glucosidase activity in the digestive tract appears to be effective way to control postprandial hyperglycemia, which has been implicated in the development of type 2 diabetes, pancreatic β-cell dysfunction, and cardiovascular disease.

Effect of FSE on blood glucose level *in vivo*

The effect of FSE on blood glucose levels after a meal was investigated in STZ-induced diabetic and normal mice. Postprandial blood glucose levels of the administered FSE were significantly lower ($p<0.05$) than those of the control and SE in diabetic mice (Fig. 3). The incremental blood glucose levels of the diabetic mice that consumed starch alone (control) were 76.0, 80.8 and 76.0 mg/dL at 30, 60, and 120 min, respectively. The incremental blood glucose levels of the mice that consumed FSE with starch were 36.3, 62.0, and 37.8 mg/dL at 30, 60, and 120 min, respectively. Consumption of FSE significantly decreased ($p<0.05$) more blood glucose levels than that of SE. Fig. 4 reveals the incremental blood glucose levels after administration of FSE with a soluble starch in normal mice. Like diabetic mice, FSE significantly reduced ($p<0.05$) the postprandial hyperglycemia caused by starch loading in comparison to the control and SE, which incremental blood glucose levels recorded as 21.8, 23.0, and 17.8 mg/dL

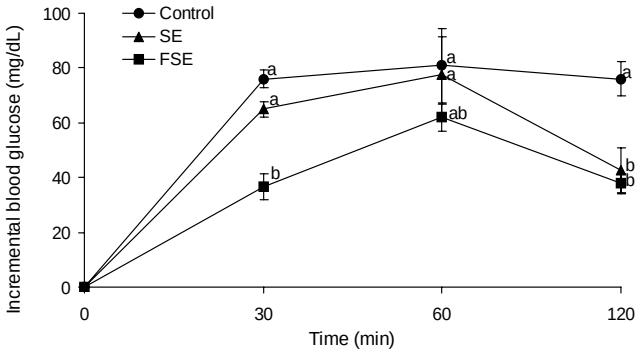

Fig. 3. Incremental blood glucose level after administration of fermented soymilk extract in STZ-induced diabetic mice. Control (distilled water), soymilk extract (SE, 500 mg/kg) and fermented soymilk extract (FSE, 500 mg/kg) were co-administered orally with starch (2 g/kg). Each value is expressed as mean±SD of seven mice (n=21). [a,b]Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test.

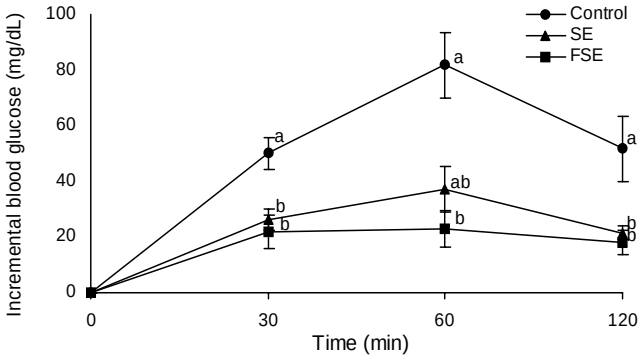

Fig. 4. Incremental blood glucose level after administration of fermented soymilk extract in normal mice. Control (distilled water), soymilk extract (SE, 500 mg/kg) and fermented soymilk extract (FSE, 500 mg/kg) were co-administered orally with starch (2 g/kg). Each value is expressed as mean±SD of seven mice (n=21). [a,b]Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test.

at 30, 60, and 120 min, respectively. The area under curve (AUC) for glucose response of administered FSE group (5,022±150.1 mg · min/dL) was significantly lower ($p<0.05$) than those of the control group (8,198 ±383.9 mg · min/dL) and SE group (6,687±457.8 mg · min/dL) in the diabetic mice (Table 2).

The treatment goal for patients with type 2 diabetes mellitus is generally agreed to maintain near-normal levels of glycemic control, both in the fasting and postprandial states (39). Postprandial hyperglycemia is the earliest metabolic abnormality to occur in type 2 diabetes (40). Postprandial blood glucose levels may be elevated in the presence of normal levels of fasting plasma glucose, constituting an early stage in type 2 diabetes (41). As shown in Fig. 3 and Fig. 4, it seems that hypoglycemic effect of FSE was more effective than that of

Table 2. Area under the curve (AUC) of postprandial glucose responses of normal and streptozotocin-induced diabetic mice

Group[1)]	AUC (mg·min/dL)	
	Normal mice	Diabetic mice
Control	6,739±183.23[a]	8,198±383.99[a]
FSE	2,225±365.31[b]	5,022±150.09[b]
SE	3,165±161.95[ab]	6,687±457.83[ab]

[1)]Control (distilled water), FSE (fermented soymilk extract, 500 mg/kg), SE (soymilk extract, 500 mg/kg) were co-administered orally with starch (2 g/kg).
Each value is expressed as mean±SD of seven mice (n=42).
[a,b]Values with different alphabets are significantly different at $p < 0.05$ as analyzed by Duncan's multiple range test.

SE on starch loading. Postprandial blood glucose peaked at 60 min after consumption of starch in the control group. FSE significantly suppressed incremental blood glucose at 30 and 60 min. These results indicate that FSE may delay absorption of dietary carbohydrates in the meal, leading to suppression of an increase in postprandial blood glucose level. It was summarized that FSE exhibited the inhibitory activities against α-glucosidase and α-amylase and it further suppressed the postprandial glucose level after starch loading in both normal and diabetic mice. Inoue et al. (42) reported that an α-glucosidase inhibitor that flattens the peak postprandial blood glucose level reduces the AUC of the blood glucose response curve. In our study, FSE decreased both incremental blood glucose in terms of the peak time as well as AUC.

Glycated hemoglobin is highly associated with a higher risk of cardiovascular disease and coronary heart disease mortality. Postprandial hyperglycemia has been known to be highly correlated with glycated hemoglobin levels and is the better predictor of glycated hemoglobin levels than fasting glucose (43). Also, postprandial hyperglycemia is strongly correlated with risk for micro- and macrovascular complications of diabetes (44). Bastyr et al. (45) demonstrated that diabetes therapy focused on lowering postprandial glucose rather than fasting glucose could be a better treatment.

In conclusion, this study demonstrated that FSE may be useful food source to treat type II diabetes via inhibiting α-glucosidase and α-amylase and the alleviation of postprandial hyperglycemia on top of the known benefits of SE. Thus, chronic consumption of FSE could be helpful in improving hyperglycemia and preventing diabetic complication. Next investigations should include the search of different pathways of FSE on diabetic control as well as determination of the component responsible for the inhibition of α-glucosidase produced by fermentation.

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