Sigumjang (fermented barley bran) water-soluble extracts inhibit the expression of adipogenic and lipogenic regulators in 3T3-L1 adipocytes

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Abstract *Sigumjang* prepared from fermented barley bran is a traditional fermented food found only in the Gyeongsang-do area of South Korea. There have been no studies reported to date despite the potential bioactivities of *sigumjang*. In this study, the anti-obesity activities of *sigumjang* extracts (SEs) during 3T3-L1 differentiation into adipocytes were investigated. SEs inhibited adipocyte differentiation by suppressing the CCAAT/enhancer binding protein- β and sterol regulatory element binding protein-1c expression in the early stage of differentiation, followed by the suppression of the peroxisome proliferator-activated receptor- γ , CCAAT/enhancer binding protein- α , and adiponectin. These changes in adipogenic markers induced inhibition of lipogenesis via down-regulation of mainly fatty acid synthase, acetyl-CoA carboxylase, fatty acid binding protein 4, and perilipin. These results were more significant in the extract of *sigumjang* fermented with isolated *Bacillus amyloliquefaciens* MFST compared to naturally fermented *sigumjang* group. SEs can be considered as a useful material for developing food with health benefits and anti-obesity properties.

Keywords: fermented barley bran foods, adipogenesis, lipogenesis, 3T3-L1 cells

Introduction

Obesity is strongly related to the development of several diseases, including cardiovascular disease, diabetes, hypertension, and dyslipidemia, and has become a global public health problem (1). Obesity is associated with abnormal growth of adipose tissue mass, caused by over expressed fat cells, and is accompanied by an alternation between energy ingesting and expenditure (2). Adipose tissues mainly comprise preadipocytes, mature adipocytes, endothelial cells, macrophages, and fibroblasts (3). Preadipocytes can differentiate into mature adipocytes, resulting in the accumulation of lipids in cells. Therefore, obesity can be ameliorated by the inhibition of adipogenesis and/or lipogenesis in adipocytes.

Adipogenesis, the process wherein preadipocytes differentiate into adipocytes, plays a critical role in obesity (4). The 3T3-L1 preadipocyte cell line is generally adopted to examine the adipogenesis mechanism as an *in vitro* model system (5). 3T3-L1 preadipocytes differentiate into adipocytes and express the genes involved in adipogenesis and lipogenesis, such as CCAAT/enhancer binding protein β (C/EBP β), sterol regulatory element binding protein-1c (SREBP-1c), peroxisome proliferator-activated receptor γ (PPAR γ),

CCAAT/enhancer binding protein α (C/EBP α), adiponectin, glucose transporter type 4 (GLUT4), fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), fatty acid binding protein 4 (FABP4), and perilipin. C/EBP β and SREBP-1c activate the transcription of C/EBP α in the early stage of adipocyte differentiation and PPAR γ , which are transcription factors that activate the expression of adipocyte genes (6,7). C/EBP α and PPAR γ are the key transcription regulatory elements expressed during adipogenesis in 3T3-L1 preadipocytes (8,9).

Sigumjang is also known as *deunggyeojang* or *gaetteokjang*, which is a traditional fermented food usually consumed in the Gyeongsang-do area of South Korea. Barley bran is the main ingredient of *sigumjang* and is a by-product of the milling process; moreover, it has been reported to help digestion (10) and inhibit cholesterol formation (11,12). In Korea, *sigumjang* is consumed as a traditional food and believed to aid digestion and treat diabetes. Despite these potential diverse bioactivities of *sigumjang*, there have been no studies on the functionality and anti-obesity effects of *sigumjang*.

In this study, we investigated the role of *sigumjang* extracts (SEs) in adipogenesis and lipogenesis in 3T3-L1 cells based on inhibition of adipocyte differentiation and lipid accumulation by measuring both mRNA and protein expression. In addition, we compared the anti-

obesity effects of naturally fermented SE (SE1) and SE fermented with isolated *B. amyloliquefaciens* MFST (SE2).

Materials and Methods

Preparation of *sigumjang* and SEs *Sigumjang* was prepared by naturally fermenting barley bran obtained in the Gyeongsang-do area of South Korea (SE1) or by inoculation of isolated *Bacillus amyloliquefaciens* MFST KCCM11719P (KCCM, Seoul, Korea) (SE2). *B. amyloliquefaciens* MFST, isolated from *sigumjang meju* collected from the Gyeongsang-do area of South Korea, has high enzymatic activities for cellulase, amylase, and protease (13). *Sigumjang meju* was prepared using steamed barley bran and water, mixed in the absence/presence of *B. amyloliquefaciens* MFST, and then fermented for 2 weeks. *Sigumjang* was prepared using *sigumjang meju*, barley, water (1:1:0.5), and NaCl (3% of total volume) and fermented at room temperature for 5 days. SEs were prepared by the general method with water. *Sigumjang* was added (fivefold of distilled water) and extracted at 65°C for 24 h with gentle shaking. The water-soluble extracts were purified by centrifugation at 6,200×g for 20 min and the supernatants were separated using Microcon YM-3 and YM-100 (Millipore, Billerica, MA, USA) for the removal of high Mw substances and salt, leaving peptides of Mw equal to 3 to 100 kDa, followed by lyophilization. The lyophilized samples were resolved in distilled water before treatment. The contents of β -glucan were measured using a β -D-glucan enzymatic assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland).

Cell culture and *in vitro* differentiation 3T3-L1 preadipocytes (KCLB, Seoul, Korea) were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% P/S at 37°C in a 5% CO₂ incubator. Confluent cells were differentiated for 3 days in a differentiation medium containing 1% penicillin-streptomycin, 10% fetal bovine serum, 10 μ g/mL of insulin, 1 μ M dexamethasone, and 10 μ M pioglitazone. The post-differentiation medium (differentiation medium excluding dexamethasone) was replaced every 2 days. After completion of cell differentiation, the cells were matured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The samples were then dissolved in distilled water and treated at a final concentration of 1 mg/mL.

Cell viability Cell viability was examined using the MTT assay (14). 3T3-L1 preadipocytes were placed into each well of a 96-well plate, followed by incubation at 37°C in a 5% CO₂ incubator for 24 h. SEs diluted with DMEM to concentrations of 0–1,000 μ g/mL were added to the wells, and the plate was incubated for an additional 24 h. MTT (final concentration of 0.25 μ g/mL in phosphate-buffered saline (PBS)) was added for 4 h, after which formazan crystals were dissolved in dimethyl sulfoxide and absorbance was measured at 540 nm using a microplate reader (Sunrise, Tecan, Vienna, Austria). Cell viability was

determined based on the ratio of absorbance of the sample-treated group to that of the control group.

Oil-Red O staining and lipid accumulation The cells were stained with Oil-Red O [16], washed twice with PBS, and fixed with 10% formaldehyde in PBS for 1 h. After discarding formaldehyde, Oil-Red O staining was performed in isopropanol for 1 h. Stained droplets were dissolved in isopropanol and then quantified by measuring absorbance at 500 nm. The results are shown as relative lipid accumulation compared to the control.

RNA isolation and semi-quantitative reverse transcription (RT-PCR)

RT-PCR of total RNA from 3T3-L1 adipocytes was performed according to a previously described method (14). Total RNA (5 μ g) was used for first-strand cDNA synthesis. Aliquots of cDNA were amplified using primers for C/EBP β : sense 5'-GACTACGCAACACA CGTGTAAC-3', antisense 5'-CAAAACCAAAACATCAACAACCC-3'; SREBP-1c: sense 5'-TGTTGGCATCCTGCTATCTG-3', antisense 5'-AGGG AAAGCTTTGGGGTCTA-3'; C/EBP α : sense 5'-TGGAC AAGAA CAGCA ACGAG-3', antisense 5'-TCACT GGTCA ACTCC AGCAC-3'; PPAR γ : sense 5'-GGAATCAGCTCTGTGGACCTC-3', antisense 5'-GGCATTGTGA GACATCCCCAC-3'; Adiponectin: sense 5'-GTGTGGCTGTGCCATCTTG AT-3', antisense 5'-GACTCAGGATGCTACTGTTGC-3'; GLUT4: sense 5'-CAGTCATTCTCATCTGGCCCT-3', antisense 5'-GTGTGGCTGTGCCATCTT GAT-3'; FAS: sense 5'-AAGCC GTTGG GAGTGAAAGT-3', antisense 5'-CAATC TGGAT GGCAG TGAGG-3'; ACC: sense 5'-TGATGTCAATCTC CCCGCAGC-3', antisense 5'-TTGCTTCTCTCTGTTTCTCCC-3'; FABP4: sense 5'-CACCTGGAAGACAGCTCCTC-3', antisense 5'-AATTTCCATCCA GGCCTCTT-3'; Perilipin: sense 5'-GATCGCCTCTGAACTGAAGG-3', antisense 5'-GATCCACATGGCCAGAGAGT-3'; GAPDH: sense 5'-CTCTA CCCACGGCAAGTTCAA-3', antisense 5'-GGATGACCTTGCCACAGC-3'. RT-PCR products were subjected to 1% agarose gel electrophoresis with 0.1 μ g/mL of ethidium bromide. Band intensities were determined by image analysis using the Bio Doc-It™ system (UVP, Inc., Upland, CA, USA).

Western blot analysis Proteins were isolated from 3T3-L1 cells treated with SEs for 3 days and 2 weeks, and total proteins were extracted from cell pellets by radio-immunoprecipitation assay (RIPA) buffer on ice. Cell lysates were centrifuged at 16,200×g for 20 min at 4°C, after which the supernatants were collected. Protein concentrations were measured using a BCA assay kit (Pierce), followed by heating at 95°C for 5 min and cooling on ice for 10 min. Proteins were separated by 10% SDS-PAGE and then transferred to an immobilon polyvinylidene difluoride membrane with transfer buffer by Trans-Blot. After blocking with skim milk (5%, 1×TBS(T)), the membrane strips were exposed to primary antibodies, followed by C/EBP α , PPAR γ , adiponectin, GLUT4, FAS, ACC, FABP4, perilipin (Cell Signaling Technology Inc., Camarillo, CA, USA), and β -actin antibodies (Biolegend, San Diego, CA, USA). After overnight incubation at 4°C, the membranes were washed three times and then incubated

Fig. 1. Effect of SEs on adipocyte differentiation and lipid accumulation. (A) Two-day post-confluent 3T3-L1 preadipocytes were differentiated with hormonal inducers (IBMX, dexamethasone, and insulin) in the absence or presence of SEs. After 3 days, all the groups were replaced with growth medium with insulin for 4 days and then growth medium. After 2 weeks of differentiation, lipid accumulation was detected by Oil-Red O staining. Representative photomicrographs (x400) are shown for each treatment group. (B) Quantitative analysis of adipocyte differentiation assessed by spectrophotometric measurement of Oil-Red O-stained adipocytes. (C) Cell viability was examined in 3T3-L1 preadipocytes incubated with SEs (1 mg/mL) for 24 h in a growth medium in the absence of MDI and analyzed by MTT assay. Blank indicates not treated. Control indicates reagent control. SE1 indicates extract of naturally fermented sigumjang. SE2 indicates extract of sigumjang fermented with *B. amyloliquefaciens* MFST. n.s. indicates not significant. Values are expressed as mean $\pm$ SD. Means with different superscripts are significantly different (* p <0.05).

with alkaline phosphatase-conjugated goat anti-rabbit IgG (Chemicon International Inc., Temecula, CA, USA). Bands were detected using a chemiluminescence kit (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions and visualized after exposure to a chemiluminescence film. Densitometric measurements of bands in the Western blot analysis were performed using the Biocapt software (version 99.01, vilber lourmat, Cedex, France) (14).

Statistical analysis Values are expressed as mean $\pm$ standard deviation. One-way analysis of variance and t-test were used for multiple comparisons. Treatment effects were analyzed using Duncan's multiple range test or Student's *t*-test by the SPSS 18.0 software. Differences are considered significant with p <0.05.

Results and Discussion

Cytotoxicity of SEs in 3T3-L1 preadipocytes

Cytotoxicity of SEs in

3T3-L1 cells was examined. Viability of 3T3-L1 cells after exposure to 1 mg/mL of SEs for 24 h was evaluated by the MTT assay. Both SE1 and SE2 did not affect the growth of 3T3-L1 cells (Fig. 1C). Morphological investigation of the 3T3-L1 cells treated with 1 mg/mL of SEs using an inverted microscope indicated that there were no morphological alterations as compared to the non-treated control group (Data not shown).

Oil-Red O staining and lipid accumulation In order to define the inhibitory effects of SEs on 3T3-L1 differentiation, 3T3-L1 cells were grown to confluence and differentiation was induced with or without SEs (1 mg/mL). After differentiation, the degree of lipid accumulation was evaluated by Oil-Red O staining (Fig. 1A). SEs significantly inhibited preadipocyte differentiation of 3T3-L1 cells. To quantify lipid accumulation, Oil-Red O-stained 3T3-L1 cells were removed and measured (Fig. 1B). The SEs treatment significantly reduced lipid droplet accumulation as compared to the differentiated control cells. Quantitative examination showed that the lipid contents of SE1 and

Panel I

Panel II

Fig. 2. Effect of SEs on mRNA expression of genes related to adipogenesis during differentiation of 3T3-L1 preadipocytes into adipocytes. Two-day post-confluent 3T3-L1 preadipocytes were differentiated in the absence or presence of SEs. Panel I is the early stage of differentiation (day 3). Panel II is the late stage of differentiation (2 weeks) (A) Representative agarose gel electrophoretic patterns of PCR products are shown. (B) mRNA expression of genes related to adipogenesis in 3T3-L1 cells was semi-quantified by RT-PCR. Densitometric signal ratios of molecules to internal control (glyceraldehydes-3-phosphatedehydrogenase [GAPDH]) were analyzed. Values are expressed as mean±SD. Means with different superscripts indicate significant differences ($p<0.05$) compared to differentiated blank cells.

SE2 were significantly different at 1 mg/mL by 12 and 14%, respectively ($p<0.05$).

Effects of SEs on mRNA expression of genes involved in adipogenesis and lipogenesis Most adipogenesis and lipogenesis mRNA markers

were significantly down-regulated by the SE treatment during early differentiation, and their inhibition was reversed in the late stage of differentiation, particularly in the case of C/EBPβ, SREBP-1c, FAS, and ACC (Fig. 2 and 3). In adipogenesis, C/EBPα, PPARγ, and adiponectin mRNA expressions were significantly reduced by the SE treatment,

Panel I

Panel II

Fig. 3. Effect of SEs on mRNA expression of genes related to lipogenesis during differentiation of 3T3-L1 preadipocytes into adipocytes. Two-day post-confluent 3T3-L1 preadipocytes were differentiated in the absence or presence of SEs. Panel I is the early stage of differentiation (day 3). Panel II is the late stage of differentiation (2 weeks). (A) Representative agarose gel electrophoretic patterns of PCR products are shown. (B) mRNA expression of genes related to adipogenesis in 3T3-L1 cells was semi-quantified by RT-PCR. Densitometric signal ratios of molecules to internal control (glyceraldehydes-3-phosphatedehydrogenase [GAPDH]) were analyzed. Values are expressed as mean $\pm$ SD. Means with different superscripts indicate significant differences ($p<0.05$) compared to differentiated blank cells.

and their inhibition was slightly reduced and continued into late differentiation. In lipogenesis, GLUT4, FABP4, and perilipin mRNA levels were significantly reduced by the SE treatment, and their

inhibition also slightly decreased and continued to decrease in the late differentiation. mRNA expressions of FAS and ACC significantly decreased in the early stage of differentiation, and their inhibition

Panel I

Panel II

Fig. 4. Effect of SEs on the protein expression of genes related to adipogenesis during differentiation of 3T3-L1 preadipocytes into adipocytes. Two-day post-confluent 3T3-L1 preadipocytes were differentiated in the absence or presence of SEs. Panel I is the early stage of differentiation (day 3). Panel II is the late stage of differentiation (2 weeks). (A) Representative patterns of Western blotting are shown. (B) Quantification of protein levels of genes related to adipogenesis in 3T3-L1 cells are shown. Densitometric signal ratios of molecules to internal control β -actin were analyzed. Values are expressed as mean $\pm$ SD. Means with different superscripts indicate significant differences ($p < 0.05$) compared to differentiated blank cells.

was reversed by late differentiation in 3T3-L1 cells. The SE2 group showed more significant inhibition of adipogenesis and lipogenesis in both the early and late stages of differentiation in 3T3-L1 cells.

Effects of SEs on protein expression of genes involved in adipogenesis and lipogenesis We examined the expression of the protein involved in adipogenesis and lipogenesis based on mRNA results. In adipogenesis, C/EBP α , PPAR γ , and adiponectin protein expressions were significantly reduced by the SE treatment and continued to decrease in the late differentiation (Fig. 4). In lipogenesis, GLUT4, FAS, ACC, FABP4, and perilipin protein levels were significantly reduced in the early stage of differentiation; furthermore, the protein levels of FAS, ACC, and perilipin continued to decrease into the late stage of differentiation (Fig. 5). For mRNA expression, FAS and ACC showed reduced expression, which can be clarified by the time gap between mRNA and protein expression. The SE2 group showed more significant inhibition of adipogenesis and lipogenesis in both early and late stages of differentiation in 3T3-L1 cells.

Obesity is considered as the increase in the size and number of adipocytes, which is controlled by nutritional, metabolic, and genetic factors (15,16). Therefore, the investigation of how certain materials influence adipocyte differentiation and lipid accumulation is important (17) to prevent obesity. Suppression of adipocyte differentiation is a useful strategy for the prevention and treatment of obesity. Recently, many research groups have focused on novel

compounds originating from natural resources with anti-adipogenesis and/or anti-lipogenesis properties. Herein we showed that SEs, water extracts of *sigumjang* prepared by fermenting barley bran, inhibit the early stage of adipocyte differentiation and late stage of lipid accumulation in 3T3-L1 cells. Prior studies have reported that the anti-adipogenic effects of several phytochemicals are related to inhibition of early adipogenic stages during differentiation (17,18).

PPAR γ , C/EBPs, and SREBP-1s play a crucial role in differentiation for adipogenesis. Herein, we observed the significant reduction of C/EBP α , C/EBP β , PPAR γ , adiponectin, and SREBP-1c mRNA and protein levels in the early stage of differentiation, and their inhibition was reversed in late differentiation particularly in the case of C/EBP β and SREBP-1c (Fig. 2 and 4). C/EBP β is an early adipogenic marker, which is significantly expressed in fully differentiated cells (19). C/EBP β synergistically induces the expression of C/EBP α and PPAR γ , which promote the expression of genes related to adipocyte differentiation (20). Prior to transcriptional activation of most adipocyte-specific genes in the early stage of adipocyte differentiation, PPAR γ , C/EBP α , and SREBP-1c are induced. These adipogenic transcription factors enhance the expression of genes such as glucose transporter 4 (GLUT4), ACC, and FAS (21). SREBP-1c is related to the expression of an endogenous PPAR γ ligand, and activation of SREBP-1c stimulates expression of FAS, ACC, and FABP (22,23). In our study, SEs significantly down-regulated the expression of genes related to adipogenesis, including C/EBP β , SREBP-1c, C/EBP α , PPAR γ , and adiponectin.

Panel I

Panel II

Fig. 5. Effect of SEs on protein expression of genes related to lipogenesis during differentiation of 3T3-L1 preadipocytes into adipocytes. Two-day post-confluent 3T3-L1 preadipocytes were differentiated in the absence or presence of SEs. Panel I is the early stage of differentiation (day 3). Panel II is the late stage of differentiation (2 weeks). (A) Representative patterns of Western blotting are shown. (B) Quantification of protein levels of genes related to lipogenesis in 3T3-L1 cells are shown. Densitometric signal ratios of molecules to internal control β -actin were analyzed. Values are expressed as mean $\pm$ SD. Means with different superscripts indicate significant differences ($p < 0.05$) compared to differentiated blank cells.

Transcription factors of adipogenesis coordinately control the expression of lipogenic genes, including FAS, ACC, GLUT4, FABP4, and perilipin. FAS, a key metabolic enzyme in lipogenesis, is identified as a terminal marker of adipocyte differentiation. FAS, which is considerably

expressed in the liver and adipose tissue, catalyzes the synthesis of saturated fatty acids in adipocytes (24,25). ACC, a rate-limiting enzyme in fatty acid synthesis, catalyzes the first reaction step, which promotes the synthesis of long-chain fatty acids and reduces fatty

Fig. 6. Schematic summary of the effects of SEs treatment on preadipocytes differentiation into adipocyte. SEs treatment inhibits the expression of C/EBP β and SREBP-1c in the early stage of differentiation, followed by inhibited expressions of C/EBP α , PPAR γ , and adiponectin. These changes in adipogenic markers down-regulate the expressions of FAS, ACC, FABP4, and Perilipin, resulting in suppressed lipid droplet formation in less matured adipocyte. Gray down-arrows in circles show inhibition effect of SEs on the molecules.

acid oxidation. Furthermore, differentiation of 3T3-L1 cells is disturbed by inhibiting ACC expression (26,27). GLUT4, a glucose transporter isoform found primarily in adipocytes and muscle cells, is involved in the active transport of glucose, and its expression is increased by C/EBPs (28,29). Perilipins are specific for adipocytes and are closely related to lipid storage droplets. Perilipins function to prevent lipolysis under basal conditions, favoring lipid deposition. Perilipins have been found to be a target gene for PPAR γ . Perilipin gene expression is increased by a PPAR γ agonist treatment of differentiated 3T3-L1 adipocytes (30-32). Our results indicate that SEs significantly reduced GLUT4, FABP4, and perilipin mRNA and protein levels, and their slight inhibition continued in the late differentiation (Fig. 3 and 5). The protein levels of major lipo-metabolic factors (FAS, ACC, and perilipin) were significantly down-regulated by the SE treatment in both the early and late stages of differentiation in 3T3-L1 cells (Fig. 5).

Recently, the effect of barley β -glucans on anti-obesity through suppressed 3T3-L1 adipocyte differentiation and lipid accumulation has been reported (33,34). β -Glucan, a non-starch polysaccharide, is found in the endosperm of grains and cell wall of the aleurone layer (35). As expected, β -glucan is normally found in *sigumjang*, which is estimated to play an important role in the anti-obesity functionality of SEs. We analyzed the β -glucan contents of the SE1 and SE2 group using kits for β -glucan analysis; the β -glucan contents of the SE2 group (2.202 g/100 g) was about 7-fold higher than that of the SE1 group (0.345 g/100 g). We confirmed that the SE2 group treated with extract of *sigumjang* fermented with isolated *B. amyloliquefaciens* MFST, which has high enzymatic activities for amylase, protease, and cellulose, showed more significant inhibition of adipogenesis and lipogenesis in both the early and late stages of differentiation in 3T3-

L1 cells compared to naturally fermented SE1 group. Based on this result, it was noted that *sigumjang* could be fermented with selected single species bacteria for its improved bioactivity. However, it was not possible to exclude the possibility of the presence of other bioactive molecules such as the phenolic compounds in *sigumjang*. This study has potential limitations as all the results were based on *in vitro* experiments with crude extracts. To further understand the bioavailability of *sigumjang*, studies with fractions of SEs including *in vivo* animal model are required.

In conclusion, SEs could inhibit the differentiation of 3T3-L1 preadipocytes by inhibiting both the mRNA and protein expressions of C/EBP β and SREBP-1c in the early stage of differentiation, followed by inhibited expressions of C/EBP α , PPAR γ , and adiponectin. These changes in adipogenic markers induced the inhibition of lipogenesis by down-regulating expressions of FAS, ACC, FABP4 and perilipin, resulting in suppressed lipid accumulation in less matured adipocyte (Fig. 6). In addition, our study showed that the extract of *sigumjang* fermented with isolated *Bacillus amyloliquefaciens* MFST has more significant anti-adipogenic effect compared to the naturally fermented *sigumjang* group. These results suggest that SEs can be a useful material for developing functional foods with anti-obesity properties.

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Disclosure The authors declare no conflict of interest.

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