# Application of High Hydrostatic Pressure for Production of Bioactive Soyasaponin from Black Soybean [Glycine max (L.)] Sprout

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**Abstract** This investigation aims to evaluate the influence of high hydrostatic pressure (HHP) treatment after germination on soyasaponin conversion and physiological activity in black soybean [*Glycine max* (L.)]. Black soybean was germinated for 4 days and subjected to 24 h at 0.1-150 MPa; the crude soyasaponin extracts were then evaluated for anti-inflammatory and anti-obesity activity. The highest crude soyasaponin content of 57.85 mg/g was found in germinated black soybean treated at 100 MPa. Soyasaponin Bb and Bc were the major B-group soyasaponins in raw black soybean; their contents in raw and germinated black soybean increased after HHP treatment. The lipid accumulation rate during 3T3-L1 adipocyte differentiation in germinated HHP-treated black soybean ranged from 93.25-65.95% at the concentration of 400 μg/mL. The highest anti-obesity effect was observed after germination and 150-MPa treatment. Black soybean sprout extracts treated at 150 MPa significantly inhibited the lipopolysaccharide-induced expression of inflammatory markers by RAW 264.7 macrophages.

*Keywords: black soybean sprout, high hydrostatic pressure, soyasaponin, anti-inflammatory activity, anti-obesity activity*

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## 1. Introduction

Soyasaponins belong to amphiphilic oleanane triterpenoid glycosides of aglycone with one or two polysaccharide chains. Differences in the aglycone compounds permit classification of soyasaponins as groups A, B, or E [1]. Soyasaponins have been reported to exert antioxidative [2,3], cholesterol-lowering [4], renin-inhibiting [5], hepatoprotective [6], and antitumor effects [7]. Most of these effects have been revealed using single compounds of group B soyasaponin; the conversion characteristics and biological activities of crude soyasaponin extracts with various treatments remain unclear.

Legumes could provide ideal nutritional components such as carbohydrates (dietary fibre), protein, fatty acids, vitamins and minerals complementary to cereal-based diets [8]. In addition, the abundant phytochemicals including soyasaponin in legumes are known to have various physiological activities such as antioxidant, anti-inflammatory and anti-proliferation abilities [9]. Black soybeans are a widely used health food, and an important source of oil, protein, and secondary metabolites including soyasaponin [10]. Among various soybean and black soybean products, sprouts, which are rich in dietary fiber, various nutrients, and bioactive components, are the valuable dietary supplements in many parts of the world that may promote health and well-being [11]. Germination is considered as an inexpensive and effective technology to improve the nutritional quality of soybean because this process triggers a sequence of metabolic changes [12]. Along with saponins and isoflavones change during germination because of accumulation and synthesis [12]. These changes in composition enhance the overall nutritional value of the soybeans and the contents of health-promoting phytochemicals [13].

High-pressure technology is used in an increasing variety of fields related to biology, and as a non-thermal food processing technique as an alternative to high-heat

processing in the food industry [14]. The use of high hydrostatic pressure (HHP) has gained prominence as a tool to perturb biochemical systems to establish the relationships between molecular structures and functions [15]. In a previous study, Kim et al. [16] reported the application of HHP technology to black soybeans to enhance their production of bioactive peptides related to inflammation makers.

The beneficial effects of soyasaponins for the reduction in incidence of chronic diseases have been linked to their modulation of obesity and inflammation factor, and the single processing methods of germination and HHP have been investigated to efficaciously enhance the functional characteristics of legumes. However, this is the first study about evaluation of physiological properties in soyasaponin rich fraction produced using high hydrostatic pressure treatment of black soybean sprout. The present study investigates the improvement of the anti-inflammatory and anti-obesity characteristics of crude soyasaponins extracted from black soybean by the combined effects of germination and HHP treatment.

# 2. Materials and Methods

## 2.1. Preparation of Black Soybean Sprout

Black soybean [*Glycine max* (L.) Merr.] were macerated in distilled water (seed:water proportion of 1:5, w/v) at 20°C ± 1°C for 24 h. The water used for macerating was dehydrated and changed every 3 h. The macerated seeds were laid down a germination tray (100 × 150 mm) comprising wet laboratory cotton stuff. They were then covered with another layer of wet cotton stuff and placed in a seed germinator (WGC 450, Dahan, Seoul, Korea) in contact with circulating water, ensuring that the seeds were constantly wet through capillary action. The seeds were left in darkness at 20°C and 95% relative humidity (RH) for 4 days [17]. The optimum germination period of 4 days was selected according to a preliminary experiment on the functional compounds and physiological activities of black soybean with germination periods varying from 0 to 6 days (data not shown).

## 2.2. High Hydrostatic Pressure (HHP) Treatment

The germinated black soybean was carried out HHP operating a warm isostatic press pressure-treatment system (WIP-L60-50-200, Ilshin Autoclave Co., Ltd., Daejeon, Korea), with the pressure chamber maintained at room temperature (RT, 20°C). The warm isostatic press applied isostatic pressure using water as the pressure medium with neither heat nor gas. The apparatus are composed of reservoir tank, high-pressure pump and vessel, control system, alarm system and safety device. Samples (20 g) of macerated black soybean and 4-day germinated black soybean were blended with 20 mL of distilled water. The mixtures were transferred to a laminated aluminum foil film (Newpack, Seoul, Korea) and heat-sealed using vacuum packaging (chamber-type vacuum package, DP-901, Dew Pack Korea Machinery Co., Seoul, Korea). HHP treatment was performed immediately after germination to prevent enzyme inactivation. The packaged samples were subjected to 0.1, 50, 100, or 150 MPa at 25°C for 24 h. All samples were dried using a freezing drier and stored at −20°C in a deep freezer (Ultra-low Temperature Freezer, MDF-393, SANYO, Akaiwa, Japan).

## 2.3. Extraction of Crude Soyasaponin

Soyasaponins were extracted according to the method of Berhow et al. [18] with slight modifications. Briefly, the samples were ground using a hammer mill and then the 100-mesh powdered samples were defatted three times with hexane at 25°C for 1 h using a shaking incubator before the extraction of crude soyasaponin. The defatted samples were extracted three times with 80% ethanol at RT for 1 h using an ultrasonic bath (SD-350H; Seong Dong, Seoul, Korea). The extracts were filtered, combined, and concentrated using a rotary evaporator under vacuum. The residue was dissolved in distilled water and extracted three times with water-saturated n-butyl alcohol. The n-butyl alcohol layer was evaporated using a rotary evaporator under vacuum and dissolved in distilled water. The dissolved extract was dried using a freeze dryer (FD5508; Ilshin BioBase, Yangju, Korea), and the dried extract was used to determine the crude soyasaponin content (mg/g soybean).

## 2.4. Analysis of Soyasaponin Composition

The soyasaponin composition of each extract was determined using a high-performance liquid chromatography (HPLC) system according to the method described by Hubert, Berger, and Dayde [19] with slight modifications. Each crude soyasaponin extract was dissolved in methanol and filtered through a 0.45-µm syringe filter (Millipore, Billerica, MA, USA). The analytical column was an octadecylsilyl (ODS) column (5 µm, 4.6 × 250 mm, Agilent Technologies). A gradient elution was employed using solvent A (water containing 0.025% (v/v) trifluoracetic acid) and solvent B (acetonitrile containing 0.025% (v/v) trifluoracetic acid). The gradient program was as follows: 0–55 min, 70% to 40% A in B (gradient); 55–56 min, 40% to 0% A in B (gradient); 56–65 min, 0% to 0% A in B (gradient); 65–66 min, 0% to 70% A in B (gradient); 66–75 min, 70% A in B (isocratic). The flow rate was kept at 1 mL/min and the injection volume was 20 µL. The UV detector was set at 205 nm. A soyasaponin standard mixture, containing soyasaponins Aa, Ab, Ba, Bb, and Bc and soyasapogenols A and B, was prepared in HPLC-grade methanol. The soyasaponin concentrations were determined by standard curves obtained by injecting different concentrations of the soyasaponin standard into the HPLC system. Peaks were verified by adding standard phenolic acids to the samples, and each peak area was calculated relative to a standard peak area. The total soyasaponin content was calculated by adding up the different soyasaponin component amounts.

## 2.5. Cell Culture and MTT Assay for Cell Viability

RAW 264.7 cells and 3T3-L1 pre-adipocytes were obtained from the Korean Cell Line Bank (Seoul, Korea) and

American Type Culture Collection (Manassas, VA, USA) respectively. RAW 264.7 cells and 3T3-L1 pre-adipocytes were maintained in separate incubators containing Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 50 μg/mL streptomycin at 37°C with 5% $CO_2$. RAW264.7 cell and 3T3-L1 pre-adipocyte death was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [20]. RAW 264.7 and 3T3-L1 pre-adipocytes were seeded at $5 \times 10^4$ cells/well in 100 μL in 96-well plates and incubated for 24 h to allow adherence, then treated with different concentrations of soyasaponin extracts (50, 100, 200, and 400 μg/mL). After incubation for 24 h, a 10-μL aliquot of 2 mg/mL MTT reagent was added to each well, and the plates were incubated at 37°C in 5% $CO_2$ humidified air for 2 h. The supernatant was carefully removed and 100 μL of dimethyl sulfoxide (DMSO) was added to each well. The resulting color was evaluated at 540 nm using an ELISA microplate reader (ELx808, Bio-Tek®, Winooski, VT, USA). Cytotoxicity was calculated as a percentage of control cell viability.

## 2.6. Measurement of Nitric Oxide (NO) Production

The measurement of nitrite using Griess reagents is widely utilized to indirectly evaluate NO production. Nitrite is a relatively stable NO oxidation product in conditioned media and can be measured by spectrophotometry [21]. Briefly, RAW 264.7 cells ($5 \times 10^4$ cells/well) were seeded in 96-well plates and incubated for 6 h at 37°C. The cells were treated with or without 0.5 μg/mL lipopolysaccharides (LPS) and the indicated concentrations of soybean protein extracts for 24 h. Then, the concentration of NO in the medium was measured using a Griess Reagent System (Promega, Madison, WI, USA). The absorbance was read at 550 nm on a microplate reader. The NO concentration was determined by comparison to dilutions of sodium nitrite as a standard.

## 2.7. Quantification of Pro-inflammatory Cytokine Production

RAW 264.7 cells ($5 \times 10^4$ cells/well) were seeded in 96-well plates and incubated for 6 h at 37°C. Cells were treated with or without 0.5 μg/mL LPS and the indicated concentrations of soybean protein extracts for 24 h. After 24 h of treatment, the culture supernatants were collected. Tumor necrosis factor α (TNF-α), interleukin (IL)-1β, and IL-6 were measured using Cymax[TM] Mouse enzyme-linked immunosorbent assay (ELISA) kits (Abfrontier, Seoul, Korea), according to the instructions provided by the manufacturer.

## 2.8. Adipocyte Differentiation and Oil Red O Staining

The 3T3-L1 cells were grown in DMEM containing 10% FBS, 100 U/mL penicillin, and 50 μg/mL streptomycin at 37°C in an incubator with 5% $CO_2$ until confluent. For adipocyte differentiation, the cells were cultured into 24-well plates at a density of $1 \times 10^5$ cells/mL, and 2-day post-confluent pre-adipocytes were stimulated for 48 h with DMEM containing 10% FBS and a hormone cocktail (0.5 mM-isobutyl-3-methylxanthine, 1 μM-dexamethasone, 5 μg/mL insulin). Cells were then cultured in DMEM supplemented with 10% FBS and 5 μg/mL insulin for another 2 days and were maintained with 10% FBS plus DMEM for an additional 2 days. To test the effect of samples on adipogenesis, the differentiation of 2-day post-confluent pre-adipocytes was induced in the presence or absence of the test sample for 4 days.

On day 6, the mature adipocytes were subjected to Oil Red O staining. After removal of the culture medium, the cells were fixed with 10% formaldehyde in phosphate-buffered saline (PBS) for 1 h at room temperature and were then washed twice with PBS. The fixed cells were stained with Oil Red O staining solution (0.3% Oil Red O in 60% isopropyl alcohol) for 20 min, followed by two washes in PBS. The stained lipid droplets were photographed using a microscope (Carl Zeiss, Jena, Germany). Finally, the dye retained in the cells was dissolved in isopropyl alcohol and was quantified by measuring the absorbance at 520 nm using a microplate reader. All samples were evaluated in triplicate and results were reported as mean ± standard deviation.

## 2.9. Statistical Analysis

Results are reported as mean ± standard deviation ($n = 3$). The statistical significance of differences among treatments was determined using the one-way analysis of variance (ANOVA) calculated by SPSS version 12 (SPSS Institute, Chicago, IL, USA) with a significance level of $P < 0.05$ determined by the Duncan multiple range test.

# 3. Results and Discussion

## 3.1. Crude Soyasaponin Content

The Influence on germination and HHP treatment of the crude soyasaponin contents in black soybean is shown in Figure 1. High hydrostatic pressure treatment after germination resulted in obvious enhanced effects on the crude soyasaponin contents of black soybean. The black soybean sprout presented 50.76 mg./g of cude soyasaponin in average, which was significantly higher than raw black soybean about 39.36 mg/g. Similarly, Chang and Han [22] observed a greatly increased enhanced the crude soyasaponin contents in general soybean cultivars applying germination, although the crude soyasaponin contents were slightly decreased during soaking processes. These increases in soyasaponins demonstrated that soyasaponins were not only were generated by biosynthetic pathways with enzyme activation as secondary metabolites, but also increased the activity of cell-wall hydrolytic enzyme and the extraction yield of soyasaponins in black soybean [23,24]. In other words, the germination effects on soyasaponin contents are based on the biosynthesis or conversion of soyasaponin to create secondary metabolites essential for plant growth.
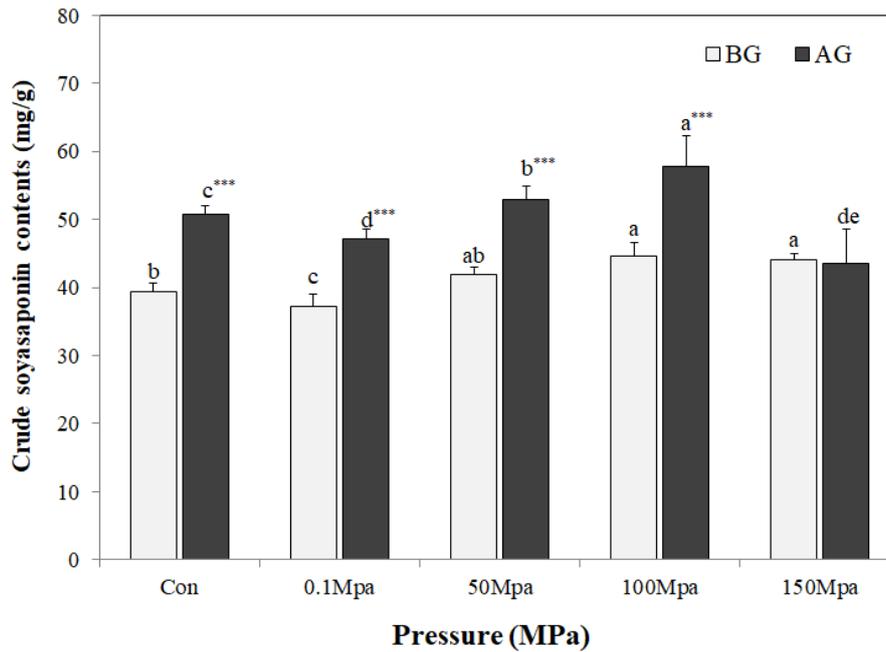
**Figure 1.** Changes in crude saponin contents with high hydrostatic pressure treatment of black soybean before(BG) and after germination(AG). Values are mean±SD of 3 replicates. Different small letters in the same items indicate a significant difference (p<0.05) among different pressure (0.1-150Mpa). *p < 0.05; **p < 0.01; ***p < 0.001; significantly different by Student's t test between before and after germination.

Also, The results of Figure 1 showed that HHP treatment contributed to improve production of soyasaponin in black soybean sprout, which varied in accordance with applied pressure. Crude soyasaponin contents of black soybean sprout increase progressively between 50.76 and 57.85 mg/g as the applied pressure increases to 100 MPa, and then decrease to 43.54 mg/g with further increases in pressure. These results may be attributed to the stimulation of enzyme activity through the denaturation of substrates at pressures lower than 200 MPa; enzyme stimulation is known to play a major role in enzymatic hydrolysis [25]. Firstly, some of the bound soyasaponins are converted into extractable free soyasaponins during germination and HHP treatment as the cell walls are hydrolyzed, allowing increased solvent influx with increasing pressure. Secondly, the soyasaponin content is dictated by the enzyme-catalyzed biosynthesis and conversion of soyasaponins into essential secondary metabolites [23,24], and this enzyme activity is much higher during germination and HHP treatment [26].

## 3.2. Soyasaponin Compositions

The influence of germination and HHP treatment on the soyasaponin compositions, with respect to the A and B groups and the seven distinct forms of soyasaponin Aa, Ab, Ba, Bb, Bc, soyasaponin A, and soyasapogenol B, in black soybean are represented in Table 1. The total soyasaponin content changes with germination and with applied pressure. The total soyasaponin contents of black soybean significantly increase from 111.44 mg/100 g to 153.32 mg/100 g after germination. In addition, the total soyasaponin contents in germinated black soybean treated by HHP range from 153.32 to 351.19 mg/100 g. The highest total soyasaponin content (351.19 mg/100 g) is observed at 100 MPa after germination. These results suggest that the total soyasaponin content is also affected synergistically by the combined effects of germination and

HHP treatment compared to germination or HHP treatment alone. Changes in the individual amounts of the seven distinct forms were monitored with combined germination and HHP treatment. Soyasaponin Aa was detected as a major A-group soyasaponin in the raw black soybean; the Aa contents are decreased with increasing applied pressure, although they are linearly increased from 1.14 mg/100 g to 3.86 mg/100 g with germination. Soyasaponin Bb, a major B-group soyasaponin in raw black soybean, showed content increases in raw black soybean and germinated black soybean increase from 94.55 and 111.98 mg/100 g to 87.46 and 128.95 mg/100 g, respectively, after HHP treatment at 100 MPa. The Bc content changes most drastically among the seven distinct forms. The Bc contents of raw black soybean and germinated black soybean increase from 9.91 and 25.85 mg/100 g to 20.44 and 241.33 mg/100 g, respectively, after HHP treatment at 150 MPa. These results are in close agreement with those reported by Shomoyamada and Okubo [27], which showed that the amounts of group A and Sg-6 saponins were reduced by 2.3- and 1.3-fold, respectively, while those of B +E saponin 2.5-fold relative to those of mature seeds. These findings show that the group A and Sg-6 saponins in mature seeds were degraded and/or translocated by germination, whereas B-group saponins were newly synthesized. Kurosawa et al. [23] reported the detection of uridine diphosphate (UDP)–glucuronic acid:soyasapogenol glucuronosyltransferase (UGASGT) activity for group B soyasaponin synthesis in microsomal fractions from germinating soybean seeds. These findings suggest that UGASGT was a specific enzyme for UDP-glucuronic acid as a donor and for soyasapogenols as acceptors, and that it was related to the biosynthesis of the sugar chain in soybean saponins. In addition, according to investigation of Gu et al. [28], B soyasaponins linked with 2,3-dihydro-2,5-dihydroxy-6-methyl-4-pyrone (DDMP) soyasaponins, which are widely distributed in legumes as the most

abundant group in soybeans, were also hydrolyzed to B soyasaponins and DDMP derivatives during sprouting. Thus, increases in B-group saponin levels are based on biosynthesis according to the activation of soyasapogenol glucuronosyltransferase, or to the conversion of B-group soyasaponin to DDMP soyasaponin. Therefore, the content of B-group soyasaponins is increased by germination and HHP treatment.

**Table 1. Changes in soyasaponin compositions of black soybean before (BG) and after germination (AG) with high hydrostatic pressure treatment**

| Germination | Pressure (MPa) | Soyasaponin Aa | Soyasaponin Ab | Soyasaponin Ba | Soyasaponin Bb | Soyasaponin Bc | Soyasapogenol A | Soyasapogenol B | Total A group | Total B group | Total soyasapogenol | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Contents of soyasaponin (mg/100 g) | | | | | | | |
| BG | Con | 1.14±0.04 | 3.29±0.39 | 1.18±0.62 | 94.75±0.05 | 9.91±0.29 | ND | 1.19±0.13 | 4.42±0.35 | 105.83±0.39 | 1.19±0.13 | 111.44±0.09 |
| | 0.1 MPa | 1.17±0.03 | ND | 0.73±0.11 | 90.11±0.82 | 11.21±0.29 | ND | 1.50±0.12 | 1.17±0.03 | 102.05±0.76 | 1.50±0.12 | 104.72±0.69 |
| | 50 MPa | ND | ND | ND | 79.03±0.65 | 12.69±0.21 | ND | 2.55±0.05 | ND | 91.72±0.86 | 2.55±0.05 | 94.27±0.81 |
| | 100 MPa | ND | ND | ND | 87.46±0.86 | 22.91±1.45 | ND | 4.45±0.63 | ND | 110.37±0.8 | 4.45±0.63 | 114.82±1.39 |
| | 150 MPa | ND | ND | ND | 86.72±0.47 | 20.44±0.01 | ND | 6.05±1.03 | ND | 107.16±0.48 | 6.05±1.03 | 113.21±0.55 |
| AG | Con | 3.86±0.05 | ND | ND | 111.98±1.39 | 25.85±0.35 | ND | 11.64±0.00 | 3.86±0.05 | 137.83±1.22 | 11.64±0.00 | 153.32±1.2 |
| | 0.1 MPa | 4.78±0.14 | ND | ND | 116.67±2.94 | 47.82±1.48 | ND | 11.08±0.48 | 4.78±0.14 | 164.49±3.33 | 11.08±0.48 | 180.34±2.97 |
| | 50 MPa | ND | ND | ND | 108.96±6.03 | 148.87±1.49 | ND | 13.27±0.30 | ND | 257.82±5.64 | 13.27±0.30 | 271.1±5.69 |
| | 100 MPa | ND | ND | ND | 128.95±0.14 | 202.9±2.46 | ND | 18.47±0.00 | ND | 331.84±2.36 | 18.47±0.00 | 350.31±2.36 |
| | 150 MPa | ND | ND | ND | 87.75±2.28 | 241.33±1.79 | ND | 22.11±0.79 | ND | 329.08±5.11 | 22.11±0.79 | 351.19±4.68 |

Values are mean±SD of 3 replicates. Different small letters in the same items indicate a significant difference (p<0.05) among different pressure (0.1-150Mpa). *p < 0.05; **p < 0.01; ***p < 0.001; significantly different by Student's t test between before and after germination.
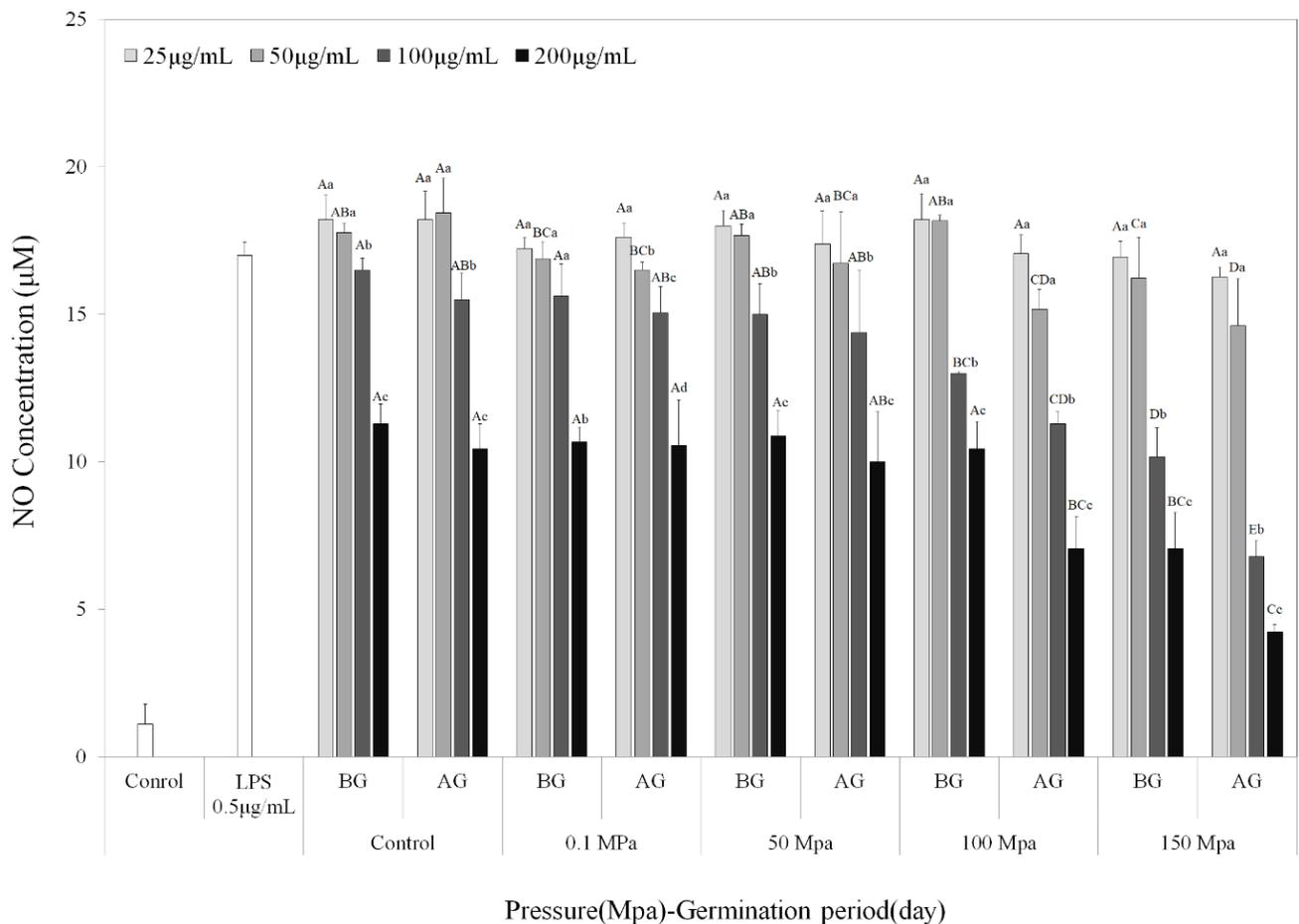


**Figure 2.** Effect of crude saponin extracts of black soybean treated by high hydrostatic pressure treatments and before(BG) and after germination(AG) on nitrate oxide concentration of RAW 264.7 cell stimulated with LPS (0.5 μg/mL) Values are mean±SD of 3 replicates. Different capital letters in the same items indicate a significant difference (p<0.05) among different pressure (0.1-150Mpa). Different small letters in the same items indicate a significant difference (p<0.05) among different germination periods of black soybean.

## 3.3. Inhibition of NO Production in RAW 264.7 Cell

The anti-inflammatory activities of crude soyasaponin extracts, including their ability to inhibit NO and pro-inflammatory cytokine (TNF-α, IL-1β, and IL-6) production, were investigated. The cytotoxic activity of the samples was determined using RAW 264.7 cells before the evaluation of anti-inflammatory effects. Cell treatments with extracts in less than 200 μg/mL concentrations did not affect RAW 264.7 cell viability (data not shown). The effects of crude soyasaponin extracts from black soybeans treated with HHP after germination on LPS-induced NO were investigated by treating RAW 264.7 cells with various concentrations of extracts and 0.5 μg/mL LPS for 24 h. The influences of germination and HHP treatment on soyasaponin extract-induced inhibition of NO production are shown in Figure 2. The NO concentration in the medium is markedly increased after treatment with 0.5 μg/mL LPS for 24 h (16.99 μM) compared to the concentration in the medium of the unstimulated control (1.12 μM). Most of the crude soyasaponin extracts inhibit NO production in a dose-dependent manner at concentrations >25 μg/mL ($p < 0.05$). The inhibitory effects of crude soyasaponin extracts on NO synthesis, an indicator of their anti-inflammatory potential, by LPS-stimulated macrophages were thus enhanced by HHP treatment after germination. The NO concentration in the medium of RAW 264.7 cells is slightly decreased from 11.28 μM to 10.44 μM in the presence of 200 μg/mL extracts from raw or germinated black soybean. Furthermore, the NO concentrations in the media of RAW 264.7 cells treated with 200 μg/mL crude soyasaponin extracts from germinated black soybean subjected to 150 MPa for 24 h ranged from 4.22 to 10.44 μM. The strongest inhibitory effect (NO concentration of 4.22 μM) is observed after treatment with crude soyasaponin extracts subjected to germination and 150 MPa for 24 h. The crude soyasaponin extract is shown to contain high amounts of soyasaponin B and soyasapogenol B, as well as an increase in the peak area of a peak corresponding to an unknown component in the HPLC analysis of crude soyasaponin extracts at 205 nm. Among soyasaponins and soyasapogenols, the anti-inflammatory characteristics of soyasaponin Ab, Ba, Bb, Bc and soyasapogenol B include the inhibition of TNF-α, IL-1β, PGE2, NO, COX-2, and iNOS productions, IκB-α phosphorylation, NF-κB activity, and iNOS enzyme activity in LPS-stimulated macrophages [29,30].

## 3.4. Inhibition of Pro-inflammatory Cytokine Production

TNF-α, IL-1β and IL-6 are important pro-inflammatory cytokines linked to the pathogenesis of many infectious and inflammatory diseases, including cancer [31]. As shown in Figure 3, LPS-stimulated RAW 264.7 cells markedly up-regulated TNF-α, IL-1β, and IL-6 production. The concentrations of TNF-α, IL-1β, and IL-6 in the media of untreated cells (7.01 ng/mL, not detected, and not detected, respectively) are increased after treatment with LPS (0.5 μg/mL) for 24 h to 635.17, 111.00, and 1.14 ng/mL, respectively. The LPS-induced increases in cytokine concentrations are inhibited in a dose-dependent manner by >50 μg/mL crude soyasaponin extracts from germinated black soybean treated at 150 MPa. Crude soyasaponin extracts from raw black soybean slightly inhibit the concentrations of TNF-α, IL-1β, and IL-6 in RAW 264.7 culture supernatants, compared to the concentrations in positive control LPS-stimulated supernatants. HHP treatment after germination enhances the pro-inflammatory cytokine inhibition behavior; treatment of LPS-stimulated RAW 264.7 cells with the highest concentration (200 μg/mL) of soyasaponin extracted from germinated black soybean subjected to 150 MPa causes reductions in TNF-α, IL-1β, and IL-6 levels to 329.67, 34.33 and 0.73 ng/mL, respectively. Kang et al. [32] reported that crude extract of soyasaponins significantly inhibited the production of pro-inflammatory cytokine TNF-α and chemokine MCP-1, the inflammatory mediator's prostaglandin E2 and nitric oxide, and the inflammatory enzymes cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) and degradation of IκB-α, an inhibitor of NF-κB, in lipopolysaccharide (LPS)-stimulated macrophages. Therefore, these results confirm that the crude soyasaponin extract of germinated black soybean show anti-inflammatory effects that HHP treatment improves. The enhanced production of soyasaponin B, soyasapogenol B, and the unknown soyasaponin, shown in HPLC, and the conversion of soyasaponin may positively affect the anti-inflammatory activity of soyasaponin extracts. Further study is required to isolate and identify the structure of active soyasaponin to clarify this correlation between soyasaponin conversion and anti-inflammatory characteristics.

## 3.5. Anti-obesity Activities in 3T3-L1 Adipocyte Cell

The cytotoxic activity of samples was determined using 3T3-L1 cells before the evaluation of lipid accumulation during 3T3-L1 adipocyte differentiation. Cell treatments with extracts of <400 μg/mL concentrations did not affect 3T3-L1 cell viability (data not shown). 3T3-L1 pre-adipocytes are among the best-characterized and most reliable in vitro models in research on new health benefit foods and agents for obesity or weight control in many studies [33]. The suppression of lipid accumulation during 3T3-L1 adipocyte differentiation has been reported as an indicator of an anti-adipogenic effect [34]. The suppression effects of lipid accumulation by crude soyasaponin extracts from black soybean treated with HHP after germination are presented in Figure 4 and Figure 5. Cellular lipid accumulation and the lipid droplets are more thoroughly suppressed by the 150-MPa HHP-treated crude soyasaponin extracts from germinated black soybean than by raw black soybean, in a dose-dependent manner. The lipid accumulation rate (% positive control) during 3T3-L1 adipocyte differentiation in germinated black soybean treated by HHP ranges from 93.25 to 65.95% at the concentration of 400 μg/mL. The highest anti-obesity effect is observed after germination and HHP treatment at 150 MPa. Plant saponins have been reported to reduce obesity by restricting calorie intake, inhibiting pancreatic lipase, and modulating certain genes involved in lipid metabolism [35,36]. Reports on the

effect of soyasaponins A and B in high fat-fed mice are rare; only total soyasaponin extracts or soyasaponin B have been studied for their hypolipidemic and hypocholesterolemic effects [37]. However, it was previously shown that soyasaponin extracts show dose-dependent inhibition of the accumulation of lipids in 3T3-L1 adipocytes [38]. Therefore, these results suggest that the crude soyasaponin extract of germinated black soybean has an anti-obesity effect that HHP treatment improves. The enhanced production of soyasaponin B, soyasapogenol B, and the unknown soyasaponin component, as well as the conversion of soyasaponin, may positively affect lipid accumulation during 3T3-L1 adipocyte differentiation.
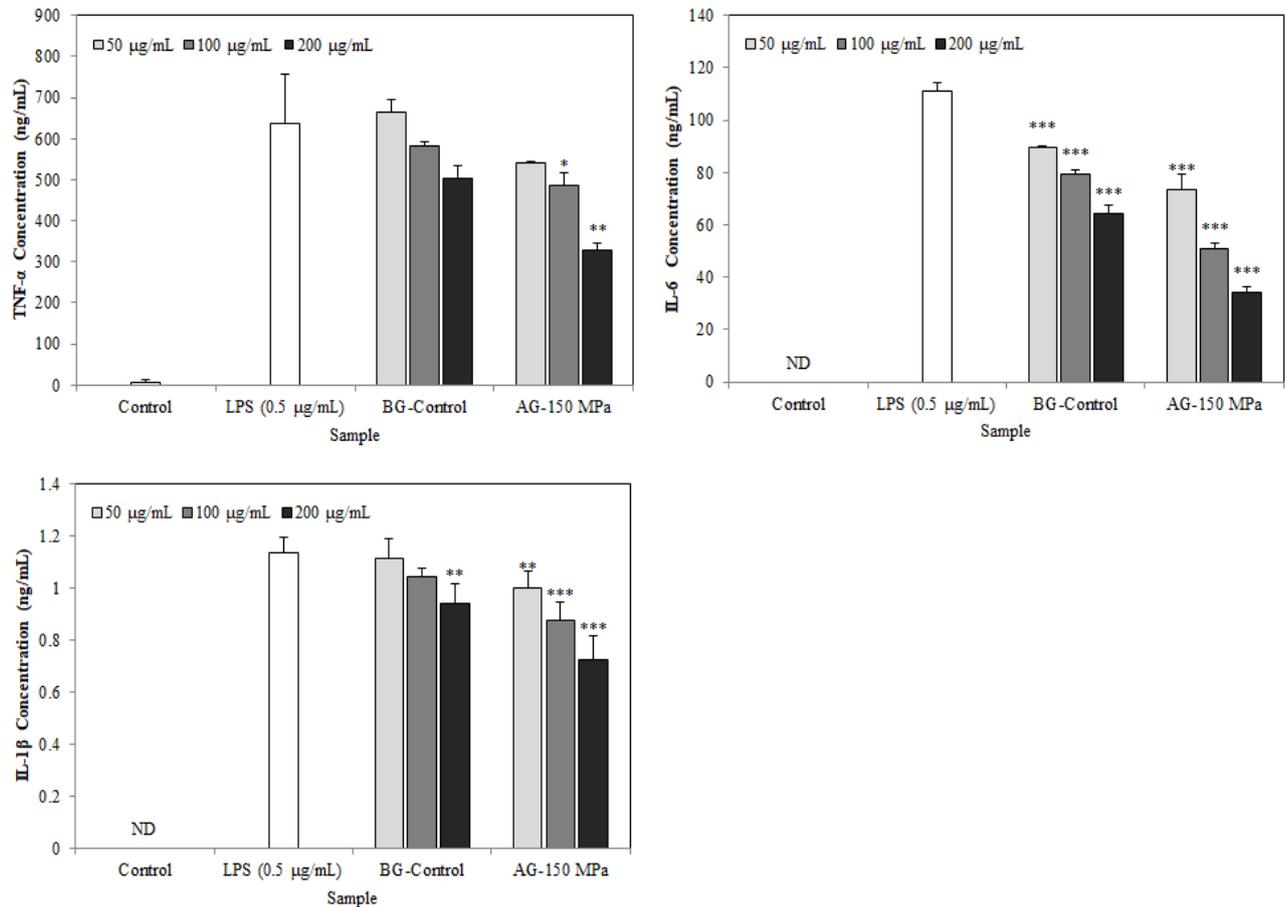


**Figure 3.** Effect of crude soyasaponin extracts of black soybean treated by high hydrostatic pressure treatments and before(BG) and after germination(AG) on TNF-α, IL-6 and IL-1β secretion of RAW 264.7 cell stimulated with LPS (0.5 µg/mL). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Significantly different by paired t-test, significantly different by Student's t-test between control stimulated with LPS (0.5 µg/mL) and samples.
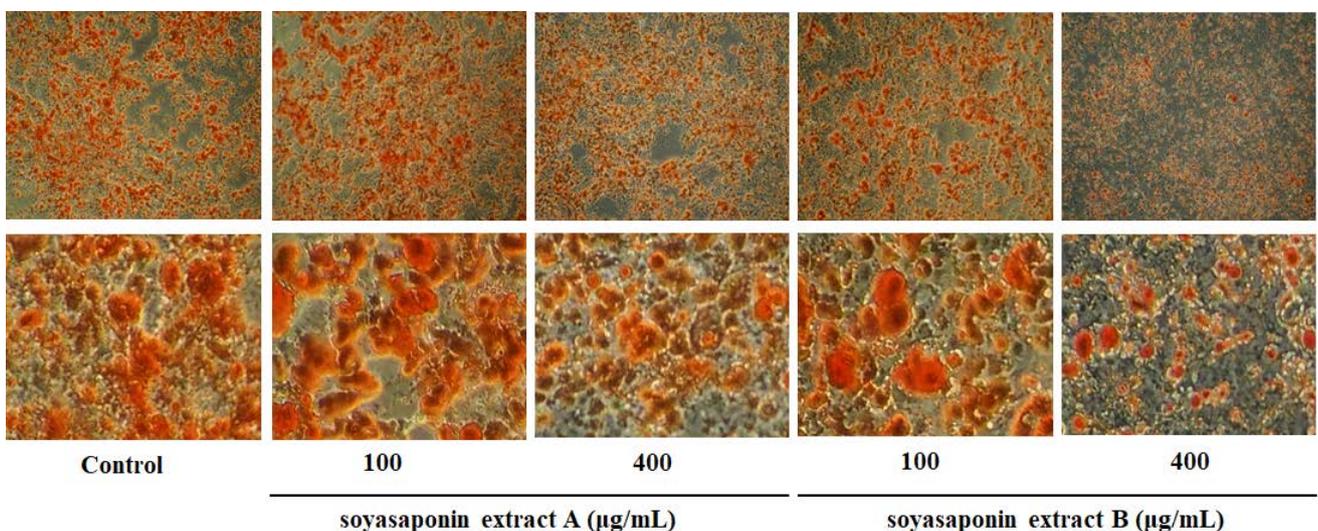


**Figure 4.** Effect of soyasaponin extracts of soybean treated by high hydrostatic pressure treatments and germination on lipid accumulation in differentiated 3T3-L1 adipocytes. (A: before germination, B: 150 MPa after germination). Cells were treated with DGSE for 4 days (days 0~4) during differentiation. Lipid content was measured on day 8 by Oil-O-Red staining method. Assays were performed in triplicates for each treatment
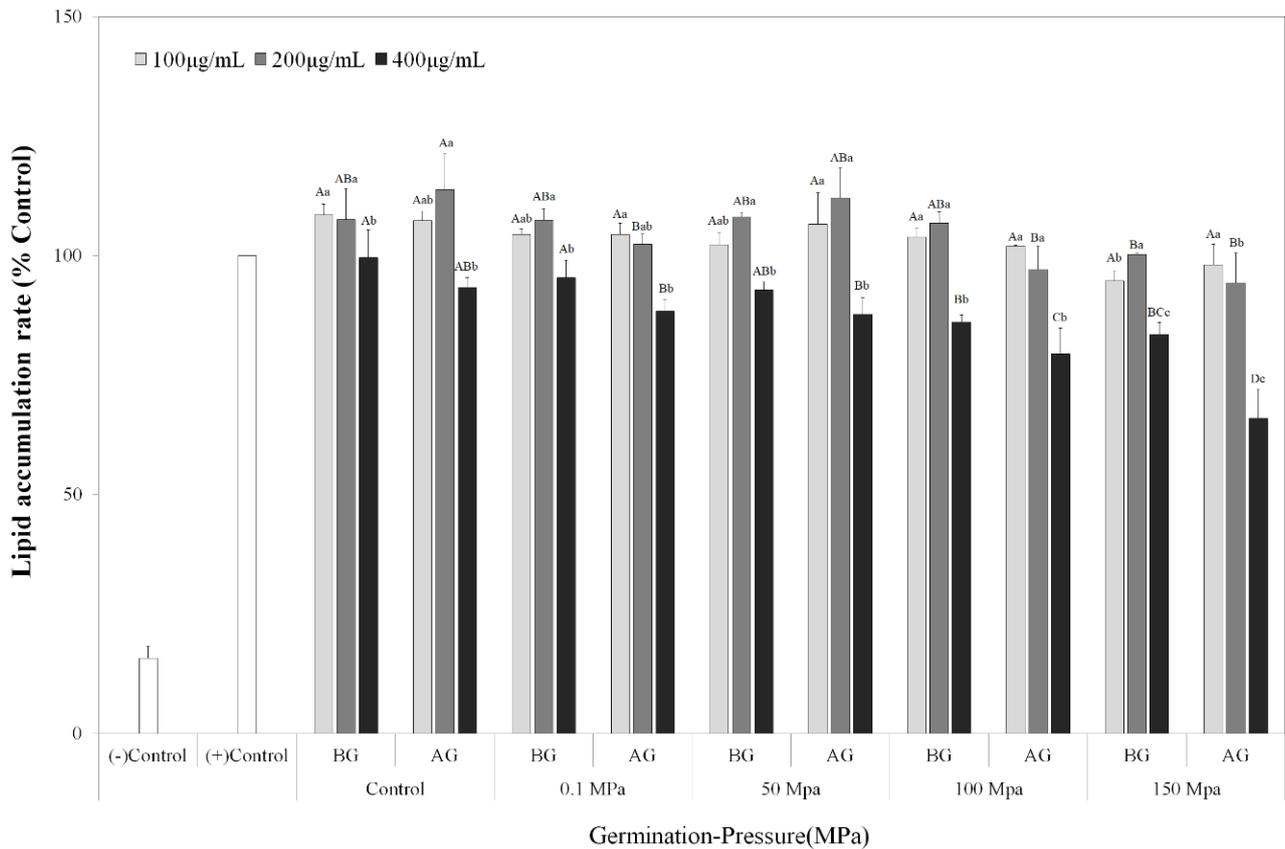
**Figure 5.** Effect of soyasaponin extracts of soybean treated by high hydrostatic pressure treatments and before(BG) and after germination(AG) on lipid accumulation in differentiated 3T3-L1 adipocytes. Cells were treated with DGSE for 4 days (0~4 days) during differentiation. Assays were performed in triplicates for each treatment. Values are mean±SD of 3 replicates. Different capital letters in the same items indicate a significant difference (p<0.05) among different pressure (0.1-150Mpa). Different small letters in the same items indicate a significant difference (p<0.05) among different germination periods of black soybean

## 3.5. Conclusion

The beneficial effects of soyasaponins in the amelioration of chronic diseases have been linked to their modulation of inflammation and obesity, and single processing steps of germination and HHP treatment have shown efficacy in increasing the functional properties of cereals and legumes. Thus, the combination of HHP treatment and germination may enhance the functionalities of soyasaponins extracted from black soybean. Indeed, combined treatment by HHP and germination yielded crude soyasaponin extracts with greater anti-inflammatory and anti-obesity properties. The highest crude soyasaponin contents were measured in black soybean subjected to 100 MPa for 24 h after germination. Soyasaponin Bb and Bc contents in raw black soybean and germinated black soybean were increased after HHP treatment at 100 MPa and 150 MPa. The highest anti-obesity effect was observed after 24 h 150-MPa HHP treatment after germination. Regarding anti-inflammatory activity, germinated soybean extracts treated at 150 MPa significantly inhibited the LPS-induced expression of inflammatory markers TNF-α, IL-1β, and IL-6 by RAW 264.7 macrophages. This study provides valuable information on the application of HHP in combination with pre-germination for improving the utilization of soyasaponin extracts as anti-inflammatory and anti-obesity agents. Further study is required to investigate the development of a high-pressure, short-time pressure and enzyme treatment method to resolve practical limits of HHP, after the identification and isolation of marker compounds from germinated, HHP-treated black soybean saponin extracts.

## Conflict of Interest

The authors have no conflicts of interest to declare.

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