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Isolation, Identification and Conversion Characteristics of Anti-inflammatory Active Saponin in Black Soybean Sprout treated by High Hydrostatic Pressure (HHP)

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ABSTRACT

Background: The use of germination and high hydrostatic pressure (HHP) processing can improve soyasaponin contents and physiological characteristics. However, no study has sought to identify the active compounds from soyasaponin extracts of black soybean treated by HHP after germination. Therefore, this study was performed to isolate and identify the anti-inflammatory active soyasaponin in black soybean sprouts treated with high hydrostatic pressure (HHP).

Methods: Black soybeans were germinated for 4 days and subjected to 150 MPa for 24 h. Crude soyasaponin extracts were separated using C18 column chromatography and the active compound was purified by semi-preparative HPLC. After isolating the active compounds, the secretion of inflammatory markers (nitric oxide, TNF-α, IL-1β and IL-6) by LPS-stimulated RAW 264.7 macrophages were measured.

Result: The anti-inflammatory compounds were identified as Soyasaponin Bb and Bc. The anti-inflammatory compounds were identified as soyasaponin Bb and Bc. Soyasaponin Bc enhanced with increasing HHP treatment time in control. However, enzyme inactivation kept soyasaponin Bc content constantly regardless of HHP treatment time. Therefore, activation of enzyme is a major factor for increasing soyasaponin BC level and anti-inflammatory activity of soybean sprout according to HHP treatment.

Key words: Anti-inflammation, Black soybean sprout, High hydrostatic pressure treatment, Soyasaponin.

INTRODUCTION

Legumes can provide ideal nutritional components such as carbohydrates, dietary fiber, proteins, fatty acids, vitamins and minerals, complementary to cereal-based diets (Guajardo-Flores *et al*., 2013). In addition, the abundant phytochemicals including soyasaponins in legumes are known to have various physiological activities such as antioxidant, anti-inflammatory and anti-proliferation abilities (Guajardo-Flores *et al*., 2013). Saponins are triterpene glycosides that occur in a wide variety of plants. Group B of soyasaponins, the predominant form of saponins, is found principally in legume seeds. In particular, Soyasaponin I (Ss-I), a major constituent of group B of soyasaponins, has been shown to possess hypocholesterolemic, anticarcinogenic and hepatoprotective properties and antioxidant activity (Gurfinkel and Rao, 2003).

Soybean sprouts are a common table food worldwide. Because of their high dietary fiber and nutrient levels and low production costs, they have become a very popular vegetable. Germinating soybeans can improve protein efficiency ratios and increase the vitamin A, vitamin C, phytoestrogen and phytochemical content (Kim *et al*., 2019). 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 (Mozhaev *et al*., 1996). Kim *et al*. (2019) reported the effects of HHP treatment after germination on the soy saponin composition, anti-obesity characteristics and regulation of inflammatory factors However, no study has sought to identify the active compounds from soyasaponin extracts of black soybean treated by HHP after germination.

Therefore, the objective of the present study was (1) to investigate the combined effect of germination and HHP on the anti-inflammatory characteristics of crude soyasaponin extracted from black soybean, regarding soyasaponin conversion, (2) to identify active ingredients by isolating bioactive compounds from crude soyasaponin extracts and

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(3) to clarify the cause of marker compound conversion in soybeans upon germination and HHP treatment.

MATERIALS AND METHODS

Preparation of germinated black soybean

Black soybean [*Glycine max* (L.) Merr.] seeds were soaked in distilled water for 24 h at 20°C ± 1°C. The soaked seeds were placed on a germination tray containing wet laboratory paper. They were then covered with another layer of wet paper and placed in a seed germinator (WGC 450; Dahan, Seoul, Korea), where they were in contact with circulating water, ensuring that the seeds were constantly wet through capillary action. The seeds were incubated in the dark at 25°C and 95% relative humidity for 4 days. These preparations were carried out at Chungbuk National University during the 2017.

HHP treatment

The germinated black soybean seeds were subjected to HHP using a warm isostatic press pressure treatment system (WIP-L60-50-200; Ilshin Autoclave Co., Daejeon, Korea), with the pressure chamber maintained at 25 ± 1°C under the same conditions as germination. Sample (20 g) were mixed with 20 mL of distilled water. The mixtures were transferred to a laminated aluminum foil film and heat-sealed using vacuum. The packaged samples were subjected to HHP treatment of 150 MPa at 25°C for 24 h.

Extraction of crude soyasaponin

Soyasaponins were extracted according to the method of Berhow *et al*. (2002). The defatted samples were extracted three times with 80% ethanol at 25°C for 1 h using an ultrasonic bath. 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).

Cell culture and cell viability assay

RAW 264.7 cells were obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 50 µg/mL streptomycin at 37°C in an incubator with a 5% CO_2 atmosphere. RAW 264.7 cell death was measured using a MTT assay (Ishiyama *et al*., 1996). RAW 264.7 cells were seeded at 5 × 10^4 cells/well in 100 µL aliquots in wells of 96-well plates and incubated for 24 h and then treated with different concentrations of the fraction isolated from crude soyasaponin extracts. After incubation for 24 h, a 10 µL aliquot of 2 mg/mL MTT solution was added to each well and the plates were incubated at 37°C in 5% CO_2 humidified

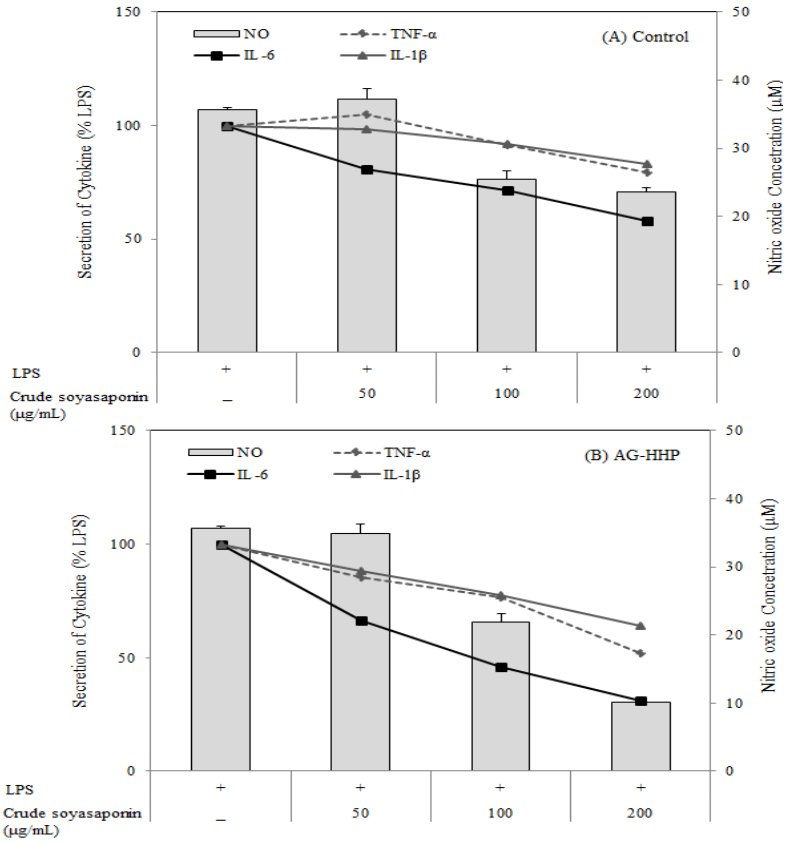


Fig 1: Effect of soyasaponin extracts of black soybean treated by high hydrostatic pressure treatments after germination (AG-HHP) on nitric oxide (NO), TNF-α, IL-6 and IL-1β secretion of RAW 264.7 cell stimulated with LPS (0.5 µg/mL).

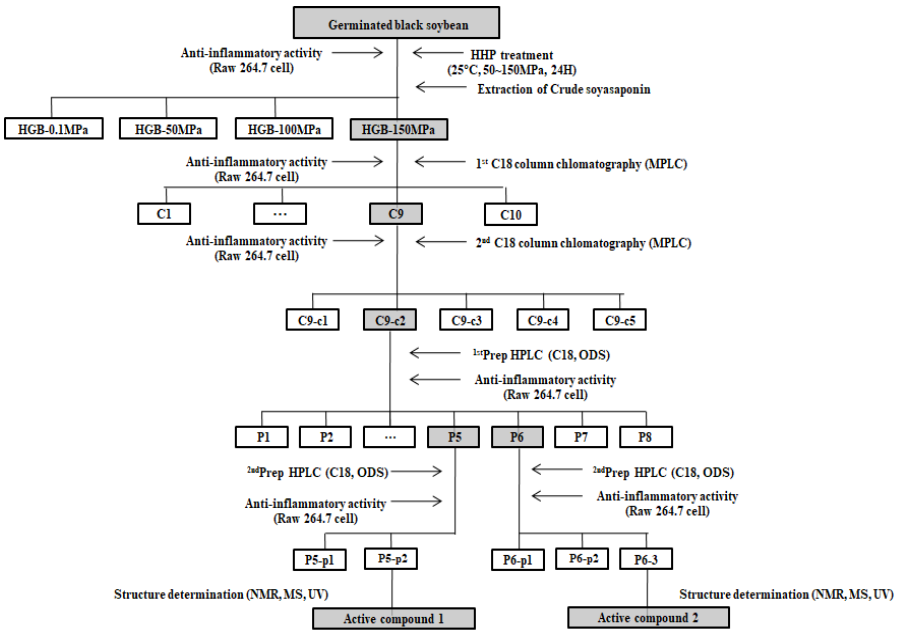
air for 2 h. The supernatant was carefully removed and 100 µL of dimethyl sulfoxide was added to each well. The resulting color was evaluated at 540 nm using an ELx808 ELISA microplate reader (Bio-Tek, Winooski, VT, USA).

Measurement of nitric oxide (NO) and pro-inflammatory cytokine production

RAW 264.7 cells (5×10^4 cells/well) were seeded in 96-well plates and incubated for 6 h at 37°C. Cells were treated with or without lipopolysaccharide (LPS, 0.5 µg/mL) and the indicated concentrations of soybean protein extract 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. Also, Levels of tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β and IL-6 were measured using Cymax Mouse ELISA kits (Abfrontier, Seoul, Korea), according to the manufacturer's instructions.

Purification and identification of anti-inflammatory compounds

Isolation and purification process is presented in Fig 2. The crude soyasaponin extracts were fractioned using C18 column chromatography on a medium-pressure liquid chromatography (MPLC) System (Key Chem Flash; WMC Coo., Ltd., Seongnam, Korea). To fractionate the saponins by polarity, a 5 mL sample (50 mg/saponin, mixed butanol) was loaded on a column packed with C18 ODS resin. The separation was performed using a mixture of water containing 0.025% trifluoracetic acid (A) and methanol (B) with an increasing amount of MeOH. The fraction with the highest anti-inflammatory activity obtained from C18 column chromatography (MPLC) was separated using a 2nd C18 column on an MPLC System. The fraction with the highest anti-inflammatory activity obtained from the 2nd C18 column chromatography (MPLC) was separated and purified by 1st semi-preparative HPLC (Younglin SP930D, Anyang, Korea). The column (Discovery® C-18 column (250 × 10 mm, 0.5 µm, Supelco) was eluted with 50% acetonitrile with 0.025% trifluoracetic acid at a flow rate of 3.0 mL/min. The fraction with the highest anti-inflammatory activity obtained from the 1st semi-preparative HPLC was purified by a 2nd semi-preparative. The structure of the purified compound was determined using several spectroscopic methods. ^{1}H nuclear magnetic resonance (NMR, 900 MHz), ^{13}C nuclear magnetic resonance (NMR, 125 MHz), ^{1}H–^{1}H correlation (COSY), heteronuclear multiple bond correlation (HMBC),



Isolation process	Sample	Yield (%)	Dose(µg/mL)	Nitric oxide (% LPS)	Cytotoxicity (% of control)
Solvent extraction	HGBS	5.79	100	61.49±0.42	No cytotoxicity
1st MPLC	HGBS-C9	1.65	100	18.96±0.74	No cytotoxicity
2nd MPLC	HGBS-C9-c2	0.60	50	24.69±1.25	No cytotoxicity
1st Prep HPLC	HGBS-C9-c2-P5	0.07	25	21.37±0.51	No cytotoxicity
1st Prep HPLC	HGBS-C9-c2-P6	0.10	25	38.68±1.67	No cytotoxicity
2nd Prep HPLC	HGBS-C9-c2-P5-p2	0.04	12.5	43.17±2.02	No cytotoxicity
2nd Prep HPLC	HGBS-C9-c2-P6-p3	0.07	12.5	35.72±0.42	No cytotoxicity

Fig 2: The schematic diagram, Isolation yield (%), cytotoxicity and nitric oxide secretion (% LPS) of the isolation process of anti-inflammatory active compound from crude soyasaponin of germinated black soybean with high hydrostatic pressure Treatment.

heteronuclear single quantum coherence (HSQC) and nuclear Overhauser effect spectroscopy (NOESY) were recorded on an Avance 400 spectrometer (Bruker) using methanol as a solvent.

Statistical analysis

All data are expressed as means ± standard deviations. Significant differences among samples determined by one-way analysis of variance using Duncan's multiple range test, with SPSS statistical program (Statistical Package for the Social Science, Ver. 12.0 SPSS Inc., Chicago, USA).

RESULTS AND DISCUSSION

Crude soyasaponin inhibited NO and pro-inflammatory cytokine production

The effects of germination and HHP on black soybean extract-induced inhibition of NO and pro-inflammatory cytokine production are shown in Fig 1. The NO concentration in the medium markedly increased after treatment with 0.5 μg/mL LPS for 24 h (35.69 μM) compared to the concentration in the medium of the unstimulated control (2.48 μM). Crude soyasaponin extracts from raw black soybean (Control) slightly inhibited the concentrations of NO in RAW 264.7 culture supernatants compared to the concentrations in positive control LPS-stimulated supernatants. HHP treatment after germination enhanced NO inhibition; treating LPS-stimulated RAW 264.7 cells with the highest concentration (200 μg/mL) of soyasaponin extracted from germinated (4 days) black soybeans subjected to HHP (150 MPa) reduced NO levels to 10.18 μM. Also, 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; treating LPS-stimulated RAW 264.7 cells with the highest concentration (200 μg/mL) of soyasaponin extracted from germinated black soybean subjected to 150 MPa caused reductions in TNF-α, IL-1β and IL-6 levels.

Isolation and purification of anti-inflammatory soyasaponin

Crude soyasaponin extracts of black soybeans treated by HHP after germination more effectively inhibited the secretion of NO and pro-inflammatory cytokines than extracts from raw black soybeans (Fig 1). Therefore, we attempted to isolate the active soyasaponin from crude soyasaponin through bioassay-guided fractionation techniques as shown in Fig 2. The HGBS was separated into nine fractions (HGBS-C1-C9) and washing fraction (HGBS-C10) using 1st C18 column chromatography and the highest NO inhibitory effects were dose-dependently observed in the C9 fraction. The HGBS-C9 fraction was

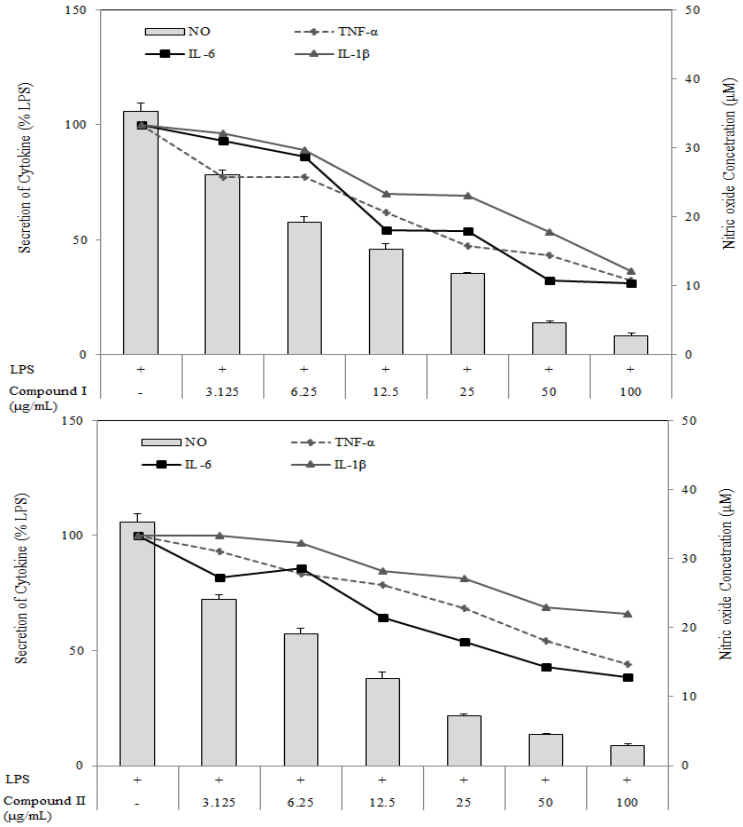


Fig 3: Effect of active compound 1 (HGBS-C9-c2-P5-p2) and compound 2 (HGBS-C9-c2-P5-p3) on Nitrate oxide, TNF-α, IL-6 and IL-1β concentration of RAW 264.7 cell stimulated with LPS (0.5 μg/mL).

further separated into four fractions (HGBS-C9-c1-HGBS-C9-c4) and for the washing fraction (HGBS-C9-c5) using 2nd C18 column chromatography, the highest NO inhibitory effects were dose-dependently observed in the C9-c2 fraction. The HGBS-C9-c2 fraction was further purified to isolate the active compound with anti-inflammatory activity using semi-preparative HPLC. The anti-inflammatory active compounds of HGBS were isolated as compound 1 (HGBS-C9-c2-P5-p2) and compound 2 (HGBS-C9-c2-P6-p3).

Identification of anti-inflammatory active compound 1 and compound 2

Active compound 1 (HGBS-C9-c2-P5-p2) and Active compound 2 (HGBS-C9-c2-P6-p3) was obtained as an amorphous powder. The purified compound was analyzed by UV, LC-MS, ^{1}H NMR, ^{13}C NMR, COSY, HMBC, HSQC and NOESY. The chemical structure of the compound is shown in Fig 5(b). The isolated compound was identified as Soyasaponin Bb and Bc and its molecular weight was 942.5188 and 912.5083. Anti-inflammatory activity of soyasaponin Bb was reported by Zha *et al*. (2011). Soyasaponin Bb inhibits TNF-α, IL-1β, PGE2, NO, COX-2 and iNOS production; IκB-α phosphorylation; NF-κB activity; and iNOS enzyme activity in LPS-induced RAW 264.7 cells. Also, The soyasaponin B group also contains soyasaponin Bc which was previously shown to significantly inhibit the production of the pro-inflammatory cytokine tumor necrosis factor (TNF)-α and chemokine monocyte chemoattractant protein (MCP)-1, the inflammatory mediators prostaglandin E2 (PGE2) and NO, the inflammatory enzymes cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) and the degradation of IκB-α, an inhibitor of nuclear transcription factor kappa B (NF-κB), in LPS-stimulated macrophages (Kang *et al*., 2005). However, no study has identified soyasaponin Bb and Bc as an anti-inflammatory active compound from germinated black soybeans treated with HHP.

Anti-inflammatory soyasaponin contents

The influence of germination and HHP conditions on the active compound 1 (soyasaponin Bb) and 2 (soyasaponin Bc) in black soybean are represented in Fig 5(a). Soyasaponin Bb contents of raw black soybean increased from 87.46 mg/100g from 128.95 mg/100 g after germination for 4 days, but this contents decreased after HHP at 150 MPa for 24 h. Soyasaponin Bc contents changed much more drastically than soyasaponin Bb, so soyasaponin Bc was selected as a marker compound of crude soyasaponin extracts (HGBS). Soyasaponin Bc contents of raw and four-day germinated samples were low, at 9.62 and 25.49 mg/100g, respectively. However, soyasaponin Bc content of raw black soybean and germinated black soybean increased to 20.43 and 243.12 mg/100g, respectively, after 150 MPa for 24 h. Kurosawa *et al.* (2002) reported that soyasapogenol glucuronosyltransferase (UGASGT) activity for group B soyasaponin synthesis was detected in the microsomal fraction from germinating soybean seed. Also, according to a study by Gu *et al*. (2017), B soyasaponins linked with DDMP (DDMP soyasaponins), which are widely distributed in legumes and are the most abundant group in soybeans, were also hydrolyzed to B soyasaponins and DDMP derivatives during sprouting. It can be concluded that increasing active compound 2 (soyasaponin Bc) occur due

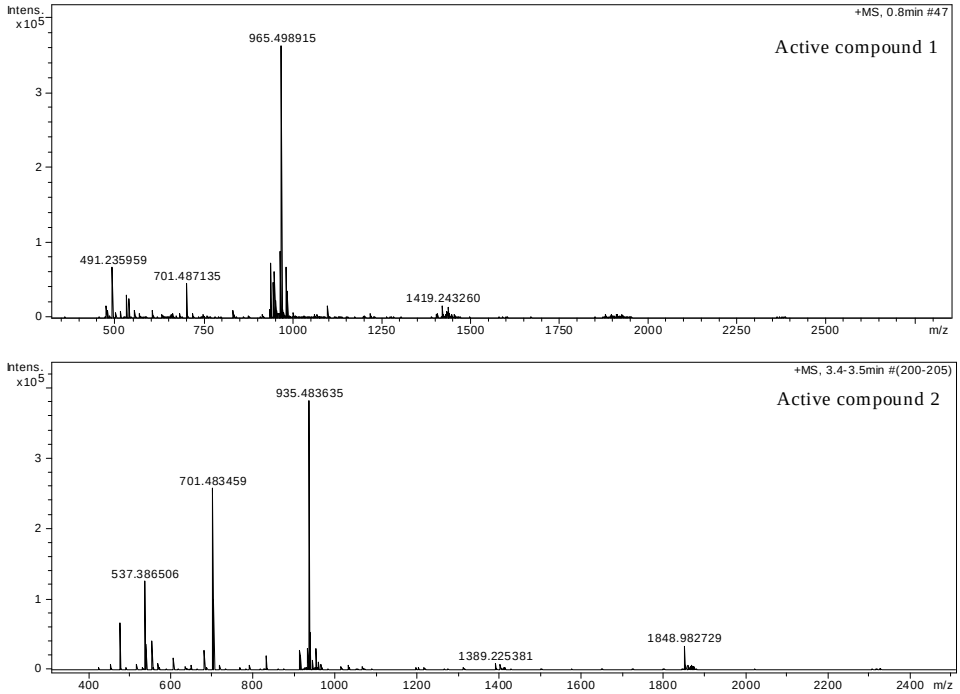


Fig 4: HPLC-MS spectrum (+MS) of active compound 1 (HGBS-C9-c2-P5-p2) and compound 2 (HGBS-C9-c2-P5-p3) isolated from crude saponin extracts treated by high hydrostatic pressure treatments (150 MPa) and germination (4 days).

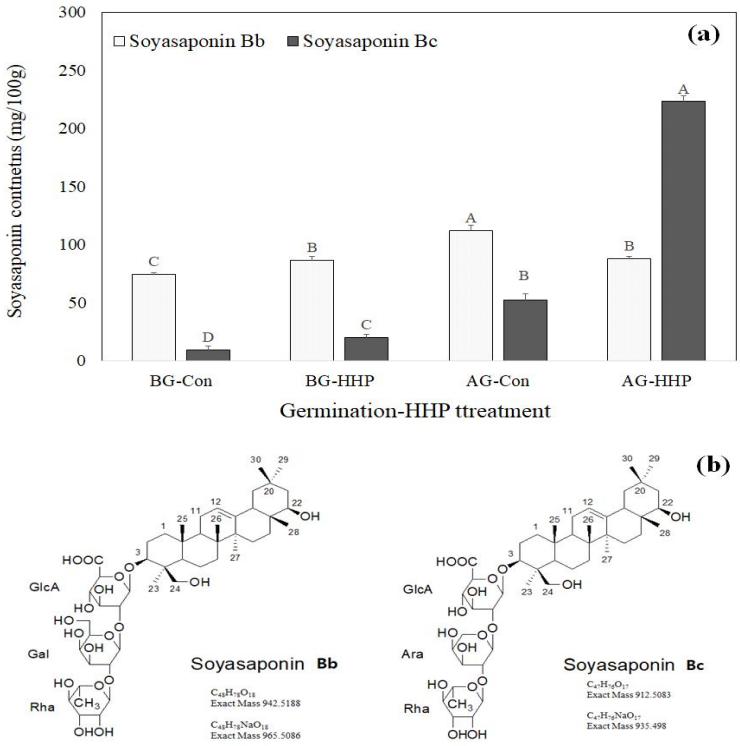


Fig 5: (a) Effect of active compound contents of black soybean with high hydrostatic pressure treatment after germination, (b) identification and chemical structure of active compound 1 (HGBS-C9-c2-P5-p2) and active compound 2 (HGBS-C9-c2-P5-p3).

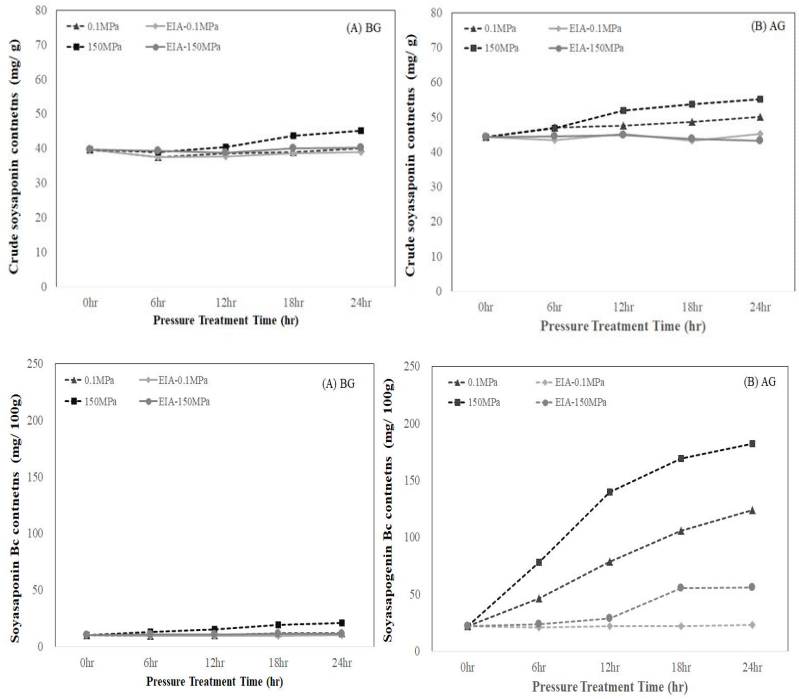


Fig 6: Changes in crude soyasaponin and active soyasaponin Bc contents of black soybean before and after germination with pressure (150 MPa for 6, 12, 18 and 24 h) and enzyme inactivation using HHP (600 MPa for 15min). *EIA: Enzyme inactivation, BG: Before germination, AG: After germination.

to biosynthesis upon the activation of soyasapogenol glucuronosyltransferase, or the conversion of B group soyasaponins to DDMP soyasaponins. These conversion and biosynthesis processes were accelerated upon HHP treatment. As a result, HHP-induced increases in soyasaponin Bc levels and biosynthesis of soyasaponin may positively impact anti-inflammatory activity.

Conversion characteristics of soyasaponin with enzyme inactivation

Changes in crude soyasaponin contents and maker soyasaponin (Bc) of germinated black soybean with high hydrostatic pressure treatment time (150 MPa for 0-24 h) and enzyme inactivation (600 MPa for 15 min) are shown in Fig 6. Factors affecting the conversion or enhancement of bioactive compound with HHP are divided into biological factors and physical factors. Biological factors including bioactive compound conversion by HHP can be explained by the activation of enzymes involved in biosynthesis (Cheftel, 1995). Physical factors that affect bioactive compound conversion by HHP can be explained by changes in cell structure and increased extraction yield (Mozhaev *et al*., 1996). Therefore, we sought to clarify the causes of compound conversion in soybeans upon germination and HHP treatment, whether biological factors or physical factors, by investigating compound (soyasaponin Bc) profiles of black soybeans before and after germination, HHP and enzyme inactivation. The influence of HHP and enzyme inactivation on soyasaponin Bc contents varied in accordance with treatment time and enzyme inactivation. Soyasaponin Bc content of germinated black soybeans increased linearly to about 22.26, 77.92, 139.78, 169.04 and 182.07 mg/100 g with increasing HHP treatment time (0, 6, 12, 18 and 24 h) in control. However, Soyasaponin Bc content was maintained a range of 22.26~55.81 mg/100 g regardless of HHP treatment time in case of enzyme in activation. Therefore, biological factors likely are a major factor in the enhanced anti-inflammatory activity and marker compound (soyasaponin) Bc of germinated black soybean with HHP. It can be concluded that increasing active compound 2 (soyasaponin Bc) levels are based on biosynthesis according to activation of soyasapogenol glucuronosyltransferase; or the conversion of B group soyasaponin to DDMP soyasaponin.

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