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Effects of High-Hydrostatic Pressure on Ginsenoside Concentrations in Korean Red Ginseng

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Abstract The effects of high-hydrostatic pressure (HHP) on the ginsenoside concentration in Korean red ginseng were investigated. HHP-pretreated Korean red ginseng samples were compared to samples produced by a conventional method. Six-year-old Korean fresh ginseng (*Panax ginseng* C.A. Meyer) samples were vacuum-packaged in polyethylene film and treated at room temperature for 1 min with HHP (200-600 MPa) and steamed at 98°C for 3 hr. Major ginsenosides of red ginseng were analyzed by HPLC. HHP-pretreated red ginseng showed a 45% higher level of total major ginsenosides than conventionally prepared red ginseng. The levels of 4 protopanaxadiol-type ginsenosides increased 34-43% and the levels of 5 protopanaxatriol-type ginsenosides increased 45-66%. Scanning electron microscopy and electrical conductivity spectrum analysis showed that HHP pretreatment damaged ginseng plant cells and increased extraction efficiencies of ginsenosides from red ginseng products.

Keywords: Korean red ginseng, high hydrostatic pressure (HHP), ginsenoside concentration

Introduction

High hydrostatic pressure (HHP) processing is a novel method of food processing wherein food is subjected to elevated pressures with or without the addition of heat to achieve microbial inactivation or to alter the food attributes in order to achieve desired qualities (1). HHP processing offers many advantages and benefits with regard to color, flavor, and nutritional quality (2). This results in a food with improved attributes.

In recent years, the HHP has been used for extraction of foods and for better functional and nutritional retention of ingredients in processed products, with improved food quality parameters (3, 4). It has many advantages, such as a shorter processing time, a higher extraction yield, a lower power consumption requirement, and fewer impurities in the extraction liquid without denaturation (5). Pressure acts as a physicochemical parameter that alters the balance of intramolecular and solvent-protein interactions (5, 6). Some authors have demonstrated the permeabilization of plant cells under pressure and showed an increase in the release of intracellular substances after pressure treatment (4, 7). The application of high pressure damages the cell wall structure, leaving the cells more permeable, which leads to significant changes in the tissue architecture resulting in increased mass transfer rates during osmotic dehydration, compared to untreated samples. When the outside pressure is quickly decreased, the instantaneous pressure difference induces structural changes in cells and quickly releases functional materials, thus increasing the extraction yield (8).

Ginseng (*Panax ginseng* C.A. Meyer) has been used extensively in Korean and Chinese medicine and has become increasingly popular in the Western world for its alleged tonic effect and possible curative and restorative properties (9). The efficacy of ginseng has been demonstrated in the central nervous system and in the cardiovascular, endocrine, and immune systems (10). In addition, ginseng and its constituents have been reported to possess anti-neoplastic, anti-stress, and antioxidant activities (11, 12).

Korean red ginseng has been developed for long-term storage and distribution. It has been reported that red ginseng has more powerful pharmacological activities than white ginseng (13, 14). The differences in the biological activities of red and white ginseng may result from a change in the chemical constituents that occur during the steaming process (15, 16). Recently, several investigators have reported new ginsenosides from red ginseng that are not usually found in raw ginseng. These are ginsenosides Rg_3, Rg_5, Rg_6, Rh_2, Rh_3, Rh_4, Rs_3, and Rf (17) and their levels in red ginseng are relatively low (18). In particular, ginsenoside Rg_2 was found to inhibit nicotinic acetylcholine receptor-mediated Na^+ influx and channel activity, resulting in an effect on the human nervous system (19). Ginsenosides Rg_3, Rg_5, and Rh_2 showed anticancer effects (20).

Not many cases of ginsenoside extraction using HHP have been reported (5, 8). Furthermore, the effects of HHP pretreatment on the extraction of ginsenosides from red ginseng have not been reported. The objective of this study was to investigate the effects of HHP pretreatment

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on the extraction of total crude saponin content and 10 major ginsenosides (Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1, Rg_2, Rg_3, and Rh_1) from Korean red ginseng.

Materials and Methods

Solvents and standards HPLC grade acetonitrile and methanol were purchased from Mallinckrodf Baker Inc. (Phillipsburg, NJ, USA). Membrane syringe filters (dia.= 13 mm, pore size = 0.45 μm) were purchased from Millipore Co. (Bedford, MA, USA). Ginsenoside Rb_1, Rb_2, Rc, Rd, Re, Rf, and Rg_1 standards were purchased from Carl Roth GmbH (Karlsruhe, Baden-Württemberg, Germany), and ginsenoside Rg_2, Rg_3, and Rh_1 standards were purchased from Alltech Associates Inc. (Deerfield, IL, USA).

Preparation of Korean red ginseng samples Freshly harvested 6-year-old Korean fresh ginseng roots (*Panax ginseng* C.A. Meyer) cultivated in Gangwha province of South Korea were used. The main roots of ginseng were washed with water and ground (dia.=2-3 mm) in a Waring® blender (37BL84; Waring Products, New Hartford, CO, USA) to reduce variations in the chemical constituents of ginseng and to prepare homogeneous samples. After HHP treatment, the ginseng sample was steam-cooked at 98±1°C for 3 hr without removing the package. The steam-cooked samples were dried after removing the package at 60°C for 4 days using a forced-convection type dryer (OF-22GW; Jeio Tech Co., Daejeon, Korea). Conventionally manufactured (control) red ginseng was also prepared using the same procedures as for the HHP-pretreated red ginseng except for the HHP pretreatment process.

High hydrostatic pressure treatment The ground ginseng was vacuum-packaged in polyethylene film (thickness = 80 μm) in a vacuum packer (Cretel 280+; Eeklo, Belgium). Pressure treatments were carried out using an isostatic pressure laboratory system (Frescal® MFP-7000; Mitsubishi Heavy Industries, Tokyo, Japan). HHP treatments (200, 400, and 600 MPa) of ground ginsengs were performed at room temperature for 1 min. Water was used as a pressure medium and targeted pressure was achieved in 3 min and the depressurization took less than 1 min.

Extraction of crude saponin A round-bottom flask fitted with a cooling condenser was used to perform saponin extraction according to the method described by Ando *et al.* (21). Extraction with 70% ethanol was carried out 3 times at 70°C. Each extraction took for 8 hr. The extract obtained was evaporated using a rotary evaporator under vacuum at 45°C.

The evaporated residue was dissolved in 100 mL of distilled water and washed with 100 mL of diethyl ether. The aqueous layer was extracted 3 times with 100 mL of water-saturated *n*-butanol. The butanol solution was washed with 100 mL of distilled water to remove impurities, thereby obtaining crude saponins. The remaining butanolic solution was transferred to the tared round bottom flask for evaporation using a rotary evaporator under vacuum at 60°C. Upon completion of the evaporation, the flask was dried at 105°C, cooled in a desiccator, and monitored until it reached a constant weight. This weight was then compared to the original weight of the empty flask. The weight difference corresponded to the amount of soluble solid (crude saponin) of the sample.

HPLC analysis of ginsenosides The levels of 10 major ginsenosides and total ginsenosides were analyzed by an HPLC method developed by Kim and Kim (22) and Lau *et al.* (23). The HPLC system used a Dionex Summit HPLC (Dionex Corp., Sunnyvale, CA, USA) with a UV detector (UVD340U), a P680 pump, an autosampler (ASI), and a column oven (TCC100). A Capcell Pak C18 MG column (4.6×250 mm, Shiseido Co., Tokyo, Japan) was also used. The detection wavelength was set at 203 nm and the solvent flow rate was held constant at 1.0 mL/min. The column temperature was fixed at 25°C using a column oven. The mobile phase used for the separation consisted of solvent A (10% acetonitrile) and solvent B (90% acetonitrile). A gradient elution procedure was used as 0-10 min 15% A, 10-70 min 82.5% A, 70-90 min 82.5% A, 90-95 min 15% A, and 95-120 min 15% A. The injection volume was 20 μL for analysis. Peak identifications were based on retention times and comparisons with injected standard samples. All solutions were filtered through 0.45 μm membrane syringe filters (Millipore Co.) before analysis. To determine calibration curves, the ginsenoside standards Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1, Rg_2, Rg_3, and Rh_1 were dissolved individually in HPLC-grade methanol, whereupon calibration standards were prepared by mixing different concentrations in appropriate quantities. The level of total ginsenosides was determined by summing the levels of the 10 ginsenosides. All values were calculated on a dry weight basis.

Confirmation of high-hydrostatic pressure by paired comparison Ten roots of 6-year-old Korean ginseng plants were washed with tap water and cut vertically into 2 parts. One part of each pair was HHP treated at room temperature for 1 min (600 MPa), and both parts of each pair underwent identical procedures for preparation of red ginseng. Both conventional and HHP-pretreated samples were steamed at 98±1°C for 3 hr and dried at 60°C for 4 days using a forced-convection type dryer, followed by extraction of saponin. The levels of total crude saponin and the 10 major ginsenosides were analyzed by HPLC and compared.

Microstructural observation of pressurized ginseng Freshly harvested 6-year-old Korean ginseng roots were washed with tap water, cut into disks (1 cm thickness), and divided vertically into 2 parts. One part of each disk was pressurized at 600 MPa for 1 min without heating and the other part was kept raw. All pressurized samples were vacuum-packaged in polyethylene film (thickness = 80 μm) before HHP treatment. HHP treated and untreated raw ginseng samples were fixed for 6 hr using Karnovsky's fixative (2% glutaraldehyde, 2% paraform-aldehyde, and 0.5% $CaCl_2$ in 0.1 M phosphate buffer solution, pH 7.4), fixed for 1 hr again using a second fixative (1% osmium tetroxide), and dehydrated using ethanol (50-100%). After fixation, samples were dried using a critical point dryer, and coated with gold. Observation was performed using a

scanning electron microscope (S-800; Hitachi, Tokyo, Japan).

Determination of impedance spectra in ginseng Five roots of 6-year-old Korean ginseng were washed with tap water, cut vertically into 2 parts, and sliced into disks (10 mm diameter, 5 mm length) using a cork borer. One part (disk) of each pair was pressurized (600 MPa) at room temperature for 1 min after vacuum-sealing in polyethylene film. The other disk was kept raw without further treatment. Impedance spectra were obtained using an impedance measurement device. The phase voltages (square wave geometry) were of equal amplitude (typically between 1 and 5 V peak to peak), and the frequency was in the range from 1 to 10 MHz. Impedance was measured in a piece of ginseng using a steel needle electrode (10 mm diameter, 10 mm length, the distance between 2 needle electrodes was 10 mm) (24). All analyses were carried out at room temperature 25±1°C.

Statistical analysis Analysis of variance was performed using Statistical Analysis System software (Version 8.01; SAS Institute., Inc., Cary, NC, USA) to test the main factor effects and their interactions. Significant differences were defined at $p<0.05$. A correlation analysis was performed using the data analysis functions of Microsoft Excel 2000™.

Results and Discussion

Effects of HHP pretreatment on Korean red ginseng HHP-pretreated and conventional red ginseng chromatograms are shown in Fig. 1. The total ginsenoside levels of both HHP-pretreated and conventional red ginseng are shown in Table 1. The level of crude saponin of HHP-pretreated red ginseng at 600 MPa was 27.61 mg, a 30% increase over conventional red ginseng. Zhang *et al.* (5) reported higher results when 6 ginsenosides (Rg_1, Re, Rb_1, Rc, Rb_2, and Rd) were studied after HHP using ethanol extraction from ginseng powder. However, this study showed higher levels of total saponin and of the 10 major ginsenosides after HHP-pretreatment. The respective levels of total ginsenosides for conventional and HHP-pretreated red ginseng ranged from 7.68 to 11.12 mg, and

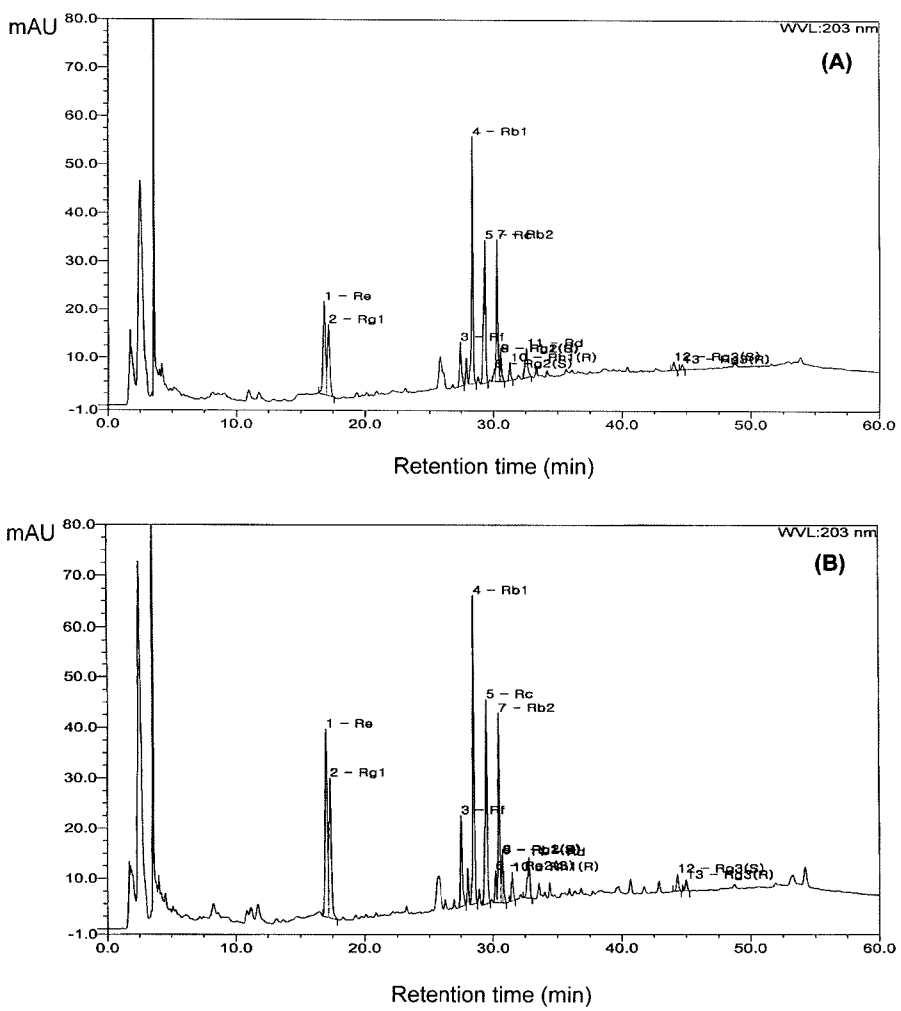

Fig. 1. High performance liquid chromatogram of 10 ginsenosides of non-HHP treated red ginseng produced by a conventional method (A) and HHP-pretreated red ginseng at 600 MPa for 1 min (B).

Table 1. The levels of ginsenosides in conventional (0.1 MPa) and HHP-pretreated (200, 400, and 600 MPa for 1 min) red ginseng samples[1)] (mg/g dry basis)

Ginsenosides		Pressure (MPa)			
		0.1	200	400	600
Protopanaxdiol	Rb_1	1.88±0.44^a	2.12±0.06^{ab}	2.38±0.35^b	2.51±0.41^b
	Rb_2	1.45±0.31^a	1.82±0.32^{ab}	1.88±0.3^{ab}	1.98±0.31^b
	Rc	1.32±0.28^a	1.44±0.19^{ab}	1.74±0.29^{bc}	1.86±0.27^c
	Rd	0.3±0.08^a	0.36±0.04^{ab}	0.38±0.02^b	0.43±0.08^b
	Rg_3(S+R)	0.05±0.01^a	0.05±0.01^a	0.06±0.03^a	0.06±0.02^a
	Re	1.2±0.4^a	1.42±0.37^{ab}	1.8±0.36^{bc}	1.99±0.16^c
	Rf	0.35±0.1^a	0.4±0.09^{ab}	0.51±0.12^{bc}	0.54±0.08^c
Protopanaxtriol	Rg_1	0.71±0.22^a	0.85±0.13^a	1.09±0.2^b	1.13±0.06^b
	Rg_2(S+R)	0.2±0.06^a	0.23±0.05^{ab}	0.29±0.07^{ab}	0.3±0.07^b
	Rh_1(S+R)	0.22±0.06^a	0.24±0.04^{ab}	0.31±0.07^{bc}	0.32±0.05^c
Total ginsenosides		7.68±1.77^a	8.91±0.88^{ab}	10.43±1.75^{bc}	11.12±1.11^c
Crude saponin		20.43±1.82^a	24.71±0.94^b	24.55±1.45^b	27.61±2.5^c

[1)]Mean±SD, n=5; Means within the same row with different superscript letters are significantly different ($p<0.05$).

HHP-pretreated red ginseng at 600 MPa had an even higher yield (44.79%) than conventional red ginseng. The levels of 4 of the 5 protopanaxadiol type ginsenosides in HHP-pretreated red ginseng increased significantly when pressure as high as 600 MPa was applied (Table 1). The levels of total ginsenosides and crude saponin pressurized at 600 MPa were higher than levels at 200 and 400 MPa (Table 1). Kim *et al.* (17) reported structural changes in ginsenosides during red ginseng manufacturing with decreases in the levels of ginsenosides Rb_1, Rb_2, Rc, Rd, Re, and Rg_1, and increases in the levels of ginsenosides Rf, Rg_3, and Rg_5 after steaming. However, our study showed different results. The level of ginsenoside Rb_1 for conventional red ginseng was 1.88 mg and the levels for HHP-pretreated red ginseng were 2.38 (400 MPa), 2.51 mg (600 MPa), respectively. The ginsenoside Rb_2 level of conventional red ginseng was 1.45 mg and for red ginseng HHP-pretreated at 600 MPa was 1.98 mg. The level of ginsenoside Rc for conventional red ginseng was 1.32 mg and for HHP-pretreated at 600 MPa was 1.86 mg, an increase of more than 40.9%. The levels of ginsenosides Rb_1, Rb_2, Rc, Rd, Re, and Rg_1, which are the main compounds of the plant (5, 25), increased by 33.5, 36.5, 40.9, 43.3, 65.8, and 59.1%, respectively, for each HHP-pretreated red ginseng sample. Our analysis confirmed that HHP pre-treatment in red ginseng processing contributes to enhanced extraction efficiencies of functional materials, such as ginsenosides, through cell structural modification.

The levels of Rg_2, Rb_2, and Rh_1, which are known to increase during red ginseng processing (14, 16, 17), showed increases with significant differences based on statistical analysis. The ginsenoside Rg_2 level of conventional red ginseng was 0.2 mg, whereas the level for HHP-pretreatment at 600 MPa was 0.3 mg, a 50% increase. The level of Rg_3 showed no significant difference between the treatments, but was slightly increased from 0.05 to 0.06 mg. Ginsenosides Rh_1, Rg_3, and Rg_2 have shown anticancer effects (26, 27) and Rh_1 has been shown to act as a phytoestrogen in breast cancer cells via activation of the estrogen receptors (26). The ginsenoside Rh_1 level of conventional red ginseng was 0.22 mg and of HHP-pretreated ginseng at 600 MPa was 0.32 mg, a 45.5% increase. Conventional extraction techniques (heat, refluxing, and soxhlet) usually require long extraction times and their efficiencies are low. Moreover, many materials are thermally unstable and may degrade during thermal extraction (8). Therefore, when choosing an extraction method, we should consider both the extraction efficiency and minimization of artificial transformation. HHP pretreatment of red ginseng should be considered for enhanced efficiency of specific ginsenoside extraction and less transformation of each ginsenoside.

Confirmation of HHP by paired comparison To determine the effect of HHP on ginsenoside compositions in the samples, paired comparisons were performed (data not shown). The crude saponin level of HHP-pretreated ginseng was higher than the level of conventional red ginseng by 4.96 mg, and the level of total ginsenosides was higher in HHP-pretreated red ginseng by 1.68 mg. Table 2 shows that the levels of crude saponin and total ginsenosides were significantly different (p=0.0029) between conventional and HHP-pretreated red ginseng. Almost all of the 10 major ginsenosides in HHP-pretreated samples showed significantly higher levels compared with conventionally prepared samples. Only Re did not show significant difference (p=0.9903).

HHP impact on Korean raw ginseng plant cells and structure The cellular structures of HHP untreated raw ginseng and HHP treated raw ginseng are shown in Fig. 3. HHP untreated raw ginseng (Fig. 2A, B, and C) showed a compact and strong ultra-structure with a glossy appearance under various magnifications. Ginseng pressure treated at

Table 2. Differences in the levels of 10 major ginsenosides, total ginsenosides, and crude saponin between red ginseng produced by a conventional method and red ginseng HHP-pretreated at 600 MPa for 1 min[1)]

Ginsenosides	Difference (mg/g dry basis)	% difference	*p*-Value
Rb_1	0.43	12.83	0.0382
Rb_2	0.49	17.96	0.0126
Rc	0.28	22.98	0.0390
Rd	0.10	38.08	0.0018
Re	0.00	0	0.9903
Rf	0.06	12.61	0.0335
Rg_1	0.13	11.66	0.0051
Rg_2	0.05	20.00	0.0010
Rg_3	0.06	164.10	0.0122
Rh_1	0.07	23.76	0.0162
Total ginsenosides	1.68	13.91	0.0029
Crude saponin	4.96	20.71	0.0029

[1)]Values are the mean of the differences of 10 paired samples.

600 MPa for 1 min showed a changed cellular structure with obvious cell damage (Fig. 2D, E, and F). The collapsed cells are still geometric, but the cell walls have lost their glossy appearance. HHP treatment destroys cell walls allowing cellular components to burst out of cells upon decompression due to osmotic pressure differences caused by infiltration of a solvent into the cell structure. As a result, the pressurized ginseng tissue has a soaked appearance.

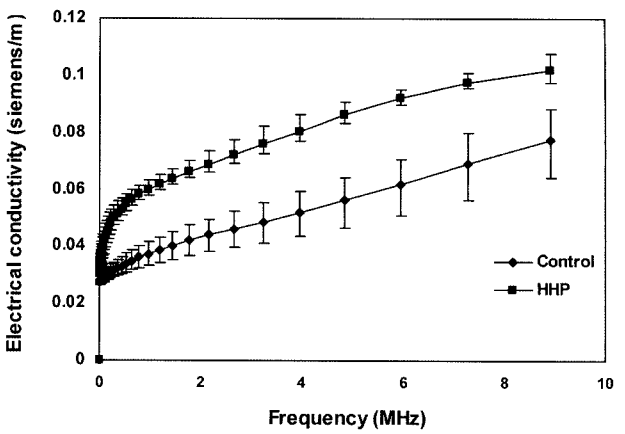

Fig. 3. Electrical conductivity spectra of HHP untreated raw ginseng (control) and HHP treated raw ginseng (HHP). HHP treatment was performed at 600 MPa for 1 min.

Typical conductivity spectra for various proportions of HHP untreated raw ginseng and HHP treated raw ginseng cells (ruptured) in the measured systems are shown in Fig. 3. The results show significant differences in conductivity between intact cells (HHP untreated) and ruptured cells (HHP treated). The frequency dependence in both measure-

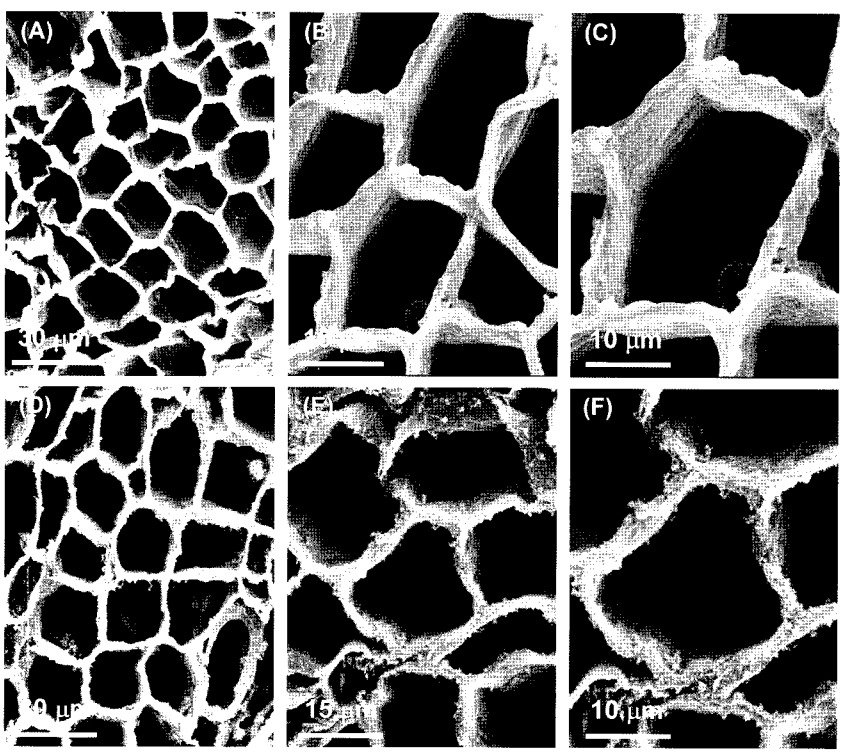

Fig. 2. Scanning electron micrographs of main body ginseng cells. (A)-(C) HHP untreated raw ginseng, (D)-(F) HHP treated raw ginseng at 600 MPa for 1 min.

ments was different. The conductivities of HHP treated raw ginseng were remarkably higher than the conductivities of HHP untreated raw ginseng.

Treatment of food with HHP results in cell permeabilisation (3), the index of which (Z_p, as measured by an electrophysical measurement based on electrical impedance analysis) increases with frequency after high pressure treatment (28). HHP treatment changes the cell permeability and enables the movement of water from inside to outside the cell (28, 29). High-pressure treatment reduces the gas space and compresses the extracellular space. As a result, the volume-related biomass concentration increases (24). Such characteristics of HHP work in favor of extraction of functional materials from plants with high water content, like ginseng (water content of ginseng is almost 70%).

In conclusion, high hydrostatic pressure pretreatment of Korean red ginseng showed no major changes in the ginsenoside profile of Korean red ginseng, but indicated a significant increase in extraction efficiencies of ginsenosides. Crude saponin and all major ginsenosides in HHP-pretreated samples showed significantly higher levels compared with conventionally prepared samples.

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