Immunostimulation Activity of a Polysaccharide from Fermented Ginseng with *Hericium erinaceum* Mycelia in Solid-state Culture

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Received September 7, 2015
Revised October 26, 2015
Accepted October 29, 2015
Published online February 29, 2016

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pISSN 1226-7708
eISSN 2092-6456

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Abstract Korean fresh ginseng was cultured with *Hericium erinaceum* mycelia (HE) in solid-state culture (SSC) to enhance the immunomodulation activity. Hot-water extracts (FG-HE-HW) of *H. erinaceum*-fermented ginseng (FG-HE) fractionated into a crude polysaccharide (FG-HE-CP) showed higher mitogenic, macrophage stimulation, and intestinal immune system modulation activities (1.52, 1.63, and 1.35x relative to a saline control at 100 µg/mL, respectively) than the crude polysaccharide from non-fermented ginseng (NG-CP; 1.35, 1.50, and 1.18x) and *H. erinaceum* mycelia alone (HE-CP; 1.26, 1.46, and 1.27x). After FG-HE-CP was further fractionated on a DEAE-Sepharose CL-6B column, the polysaccharide fraction (FG-HE-CP-III) exhibited larger activity increases than any subfraction from NG-CP or HE-CP. FG-HE-CP-III mainly consisted of Ara, Gal, Glc, and GalA (molar ratios of 0.45:1.00:0.51:0.73). NaIO₄ oxidation decreased the activities of FG-HE-CP-III (51.0–76.9%). The polysaccharide from *H. erinaceum*-fermented ginseng using SSC plays an important role for enhancement of immunomodulation activities of fresh ginseng.

Keywords: *Hericium erinaceum*, fermented ginseng, solid-state culture, immunostimulation, polysaccharide

Introduction

Panax ginseng C.A. Meyer is Korean representative ginseng and has been taking a long time to traditional herbal medicine. It is administered to patients suffering chronic diseases to improve their debilitated condition by homeostasis maintenance, and activation of protection system, such as immune system (1). Commercially available ginseng is classified into fresh, white, and red ginseng. White ginseng (WG) is made by peeling fresh ginseng and drying without steaming, whereas red ginseng (RG) is made by steaming followed by drying. RG exhibits more pharmacological activities than WG (2,3). Differences in biological activities of WG and RG may result from changes in chemical constituents that occur during steaming treatments. Hwang *et al.* (4) reported that heat processing induced structural changes that might enhance biological activities and effectiveness. New ginsenosides have been reported in RG that are not usually found in WG or fresh ginseng (5,6). Thus, steaming and drying processes may provide physiological ingredients and increase activities of RG.

RG extracts are more popular as functional foods for immunostimulation, and as a tonic, by more people than WG extracts.

However, heating can degrade thermally unstable nutrients and pharmacological ingredients. Although the main active components of ginseng is generally known as ginsenosides, peptides and polysaccharides has been widely studied in recent years. In particular, ginseng polysaccharide has been reported to play an important role of immunomodulating activity and anticancer activity (7,8). Active ginseng polysaccharides can be degraded by thermal and/or pressurized processes in steaming for RG preparation, and physiological activities and yields of polysaccharides can be decreased in comparison with fresh ginseng.

Solid-state culture (SSC) is the fermentation process of solids with the appropriate water content for tissue softening to grow microbes into the interior. Since SSC does not require the complex maintenance of fermentation condition and lots of energy input, it has many advantages for industrial utilization compared with submerged fermentation. In addition, SSC is useful process to enhance physiological activities by structural change of active component, and to produce high-value substances from common agricultural products or waste materials (9). In previous studies (10), fresh ginseng was fermented using Basidiomycetes, and immunological activities were upgraded.

A new approach was envisaged for development of a more efficient and dependable method for culture using the bearded tooth mushroom *Hericium erinaceum*.

The aim of this study was to enhance the immunomodulation activity of Korean fresh ginseng using SSC. Fresh ginseng was cultured with *H. erinaceum* for preparation of *H. erinaceum*-fermented ginseng, which was fractionated into a crude polysaccharide with an immunostimulation activity. The active polysaccharide was isolated and characterized for development of a new functional material.

Materials and Methods

Mycelia and ginseng *H. erinaceum* mycelia and fresh ginseng (*P. ginseng* of 5 year cultivation) were obtained from the Agricultural Research and Extension Services of Gyeonggi, Korea, and the Farming Association of Jeungpyeong-gun in Chungbuk, Korea, respectively, in April of 2013. Ginseng had been stored in a freezer at -20°C for preservation of quality. After fresh ginseng was decocted 3x at 95°C using distilled water, the resulting filtrate was centrifuged at 7,600xg for 30 min (Hanil Scientific Industrial, Incheon, Korea) and concentrated (Buchi Labortechnik AG, Flawil, Switzerland) to a ginseng extract (65°Bx) for use as a seed culture medium for *H. erinaceum* mycelia.

Preparation of *H. erinaceum*-fermented ginseng using SSC A mushroom complete medium (MCM) was previously optimized as a basal medium for a seed culture of *H. erinaceum* mycelia (10). MCM contained 20 g of glucose, peptone/yeast extract (2 g each), and K_2HPO_4 , KH_2PO_4 , and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1, 0.4, and 0.5 g, respectively) per L. Mycelia were cultured in a flask containing MCM supplemented with a 5% (v/v) ginseng extract in a shaking incubator (SI-400R; Jeio Tech., Daejeon, Korea) at 30°C and pH=5.5 for 6 days (10). Washed fresh ginseng was dried for 3-4 days to control the moisture content and soften the tissue, then a 10% (w/w) ratio of dried ginseng and brown rice powder as a nutrient was added to the dried ginseng. After autoclaving (Daihan Scientific, Wonju, Korea), a mycelia seed at 10% (v/w) was inoculated into the ginseng and cultured in multi-room incubator (VS-3DM; Vision Scientific, Daejeon, Korea) at 30°C for 30 days, followed by lyophilization (Samwon Freezing Engineering Co., Busan, Korea) into fermented ginseng (FG-HE) (Table 1).

General methods The phenol-sulfuric acid (11), *m*-hydroxybiphenyl (12), and Bradford's (13) methods using Bio-Rad dye (Bio-Rad, Hercules, CA, USA) were followed for determination of total carbohydrate, uronic acid, and protein contents, respectively, using respective standards of galactose (Gal), galacturonic acid (GalA), and bovine serum albumin (BSA). The neutral monosaccharide composition of polysaccharides was analyzed following the alditol acetate derivative method (14) with slight modification. Briefly, the polysaccharide fraction was hydrolyzed using 2 M trifluoroacetic acid (TFA) (Sigma-

Aldrich Co., St. Louis, MO, USA) for 1.5 h at 121°C , and the hydrolyzed aldose component was substituted to alditol acetates by NaBH_4 (Sigma-Aldrich Co.) reduction and acetic anhydride (Sigma-Aldrich Co.) acetylation. Resulting alditol acetates were analyzed following the method of Jones and Albersheim (14) using an M600D system gas liquid chromatography apparatus (GLC) (Young Lin Instrument Co., Anyang, Korea) equipped with a flame ionization detector (Young Lin Instrument) and a Supelco SP-2380 capillary column (30 m $\times$ 0.25 mm i.d., 0.2 μm film) (Supelco, Bellefonte, PA, USA). The polysaccharide was hydrolyzed using 2 M TFA, followed by NaBH_4 reduction and acetic anhydride acetylation. Resulting reduced hydrolysates were alternatively loaded onto Dowex-1 resin (Dow Water & Process Solutions, Edina, MN, USA) for analysis of the acidic monosaccharide composition. Adsorbed aldonic acids were eluted using 1.0 N HCl and the eluted fraction was evaporated to dryness at 40°C in a stream of filtered air. Resulting lactones were reduced and acetylated under neutral conditions. Resulting carboxyl-reduced alditol acetates were analyzed following the procedure of Zhao *et al.* (15) using GLC as above.

Fractionation of the crude polysaccharide from *H. erinaceum*-fermented ginseng After 100 g of FG-HE was directly homogenized (Ultra-Turrax® T-50; Janke & Kunkel IKA-Labortechnik, Staufen, Germany), the homogenate was decocted 3x using 2 L of distilled water to half the original volume. Combined extracts were centrifuged at 7,600xg at 4°C for 30 min (Herolab GmbH Laborgerate, Wiesloch, Germany) to remove insoluble materials, and the supernatant was lyophilized for preparation of hot-water extracts (FG-HE-HW). Four volumes of ethanol were added to dissolve FG-HE-HW, then the resulting precipitate was dialyzed (Spectra/Por 4, MWCO 12-14,000; Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) against distilled water. After insoluble materials were removed using centrifugation, the non-dialyzable portion was lyophilized to a crude polysaccharide fraction (FG-HE-CP).

Isolation of an immunostimulating polysaccharide from FG-HE-CP When 1 g of FG-HE-CP was loaded onto a DEAE-Sepharose CL-6B (Cl^- form) column (4.0x40 cm) (Amersham Biosciences, Uppsala, Sweden), a non-adsorbed fraction (FG-HE-CP-I) with a 9.28% yield of the FG-HE-CP loading amount was eluted using distilled water. A step-wise gradient of 0.1, 0.2, 0.3, 0.4, 0.5, and 1.0 M NaCl was used for elution of the 6 absorbed fractions, which were then dialyzed against water and lyophilized. Adsorbed fractions were obtained as lyophilizates of 0.1 M NaCl/FG-HE-CP-II with a 9.64% yield, 0.2 M/FG-HE-CP-III with a 17.56% yield, 0.3 M/FG-HE-CP-IV with a 23.40% yield, 0.4 M/FG-HE-CP-V with a 11.90% yield, 0.5 M/FG-HE-CP-VI with a 3.48% yield, and 1.0 M/FG-HE-CP-VII with a 2.44% yield.

Chemical and enzymatic treatments of the active polysaccharide fraction The active polysaccharide (FG-HE-CP-III, 10 mg) was oxidized using 10 mL of 25 mM NaIO_4 in a 50 mM acetate buffer at pH 4.5 at

4°C for 96 h (16). Then, 1 mL of ethylene glycol was added, followed by stirring (Misung Scientific Co. Ltd., Yangju, Korea) for 1 h at room temperature to stop the reaction. After dialysis using a Spectra/Por 4 (Spectrum) for 72 h, the non-dialyzable portion was concentrated to half the original volume. Then, 50 mg of NaBH₄ was added, followed by stirring for 8 h at room temperature, followed by neutralization using concentrated acetic acid. The solution was dialyzed against distilled water and the non-dialyzable portion was lyophilized to yield NaIO₄-oxidized FG-HE-CP-III with an 81.7% yield. For enzymatic treatment, 10 mg of actinase E (Kaken Pharmaceutical Co. Ltd., Tokyo, Japan) was added to 10 mg of a FG-HE-CP-III solution in 10 mL of a 50 mM Tris-HCl buffer at pH 7.9 containing 10 mM CaCl₂, and the mixture was incubated (JSResearch Inc., Gongju, Korea) at 37°C for 48 h (17). After the reaction was stopped by neutralization with 0.1 N HCl, the solution was dialyzed using Spectra/Por 4 against distilled water for 72 h, and the non-dialyzable portion was lyophilized to yield pronase-digested FG-HE-CP-III with an 85.4% yield.

Mice and cell culture Pathogen-free female 6 week old C3H/He mice were purchased from Orient Bio (Seongnam, Korea), and 6 week old female BALB/c and ICR mice were obtained from Nara Biotech Co., Ltd. (Seoul, Korea). Mice were housed in an air-conditioned room at 24±2°C with a relative humidity of 55±10% under a 12-h light-dark cycle and fed a standard laboratory diet (Samyang Co. Ltd., Incheon, Korea) with water *ad libitum*. Animal experiments were approved and performed in accordance with Guidelines for Care and Use by the Animal Research Committee of Korea University (KUIACUC-2014-248, Seoul, Korea). Peritoneal macrophages, splenocytes, and Peyer's patch primary cells from mice were cultured (VS-9108MS; Vision Scientific) in RPMI-1640 (Gibco-BRL Co., Grand Island, NY, USA) supplemented with fetal bovine serum (FBS) (Cell Culture Laboratories, Cleveland, OH, USA), 100 U/mL penicillin (Flow Laboratories, Irvine, Scotland), 100 µg/mL streptomycin (Flow Laboratories), and amphotericin B (Flow Laboratories) at 37°C in a 5% CO₂ atmosphere.

Mitogenic activity of splenocytes After splenic lymphocytes (5×10⁶ cells/well) prepared from spleens of BALB/c mice were cultured with hot-water extract or subfraction in 96 well culture plates (MaxiSorp™ F96; Nunc®, Roskilde, Denmark) for 48 h, lymphocyte proliferation was assayed using a cell counting kit (CCK-8; Dojindo Laboratories, Kumamoto, Japan) (17). The CCK-8 kit provides a convenient assay based on use of highly water-soluble tetrazolium salt. WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, mono-sodium salt] produces a water-soluble formazan dye in direct proportion to the number of living cells upon activity of dehydrogenases in cells. The detection sensitivity of CCK-8 is also higher than for other tetrazolium salts, such as MTT, XTT, MTS and WST-1 (17). Four h prior to culture termination, 20 µL of a CCK-8 solution was added to each well, then cells were continuously incubated for 4-24 h, after which the culture was measured at 450

nm using a microtiter plate reader (Sunrise; Tecan, Grödingen, Austria). The mitogenic activity was expressed as a relative percentage of splenocyte growth to a saline control incubated without hot-water extract or subfraction.

Macrophage stimulation assay ICR mice were injected interperitoneally with 1 mL of 3% thioglycollate medium (TG). After 3 days, macrophage cells were collected from the peritoneal cavity and prepared by washing with cold RPMI 1640 medium containing 5 mM HEPES, penicillin, and streptomycin. A 200 µL aliquot of the cell suspension (1×10⁶ cells/mL) was cultured in a flat-bottomed 96-well plate for 2 h at 37°C in a humidified atmosphere of 5% CO₂-95% air. Non-adherent cells were removed by washing with RPMI-1640 medium, then adherent cells were prepared into a macrophage monolayer (18). The macrophage stimulation activity was measured following the procedures of Suzuki *et al.* (19) with slight modification. After macrophage monolayer cells (1×10⁵ cells/mL) were cultured with samples for 24 h, cells were solubilized using addition of 25 µL of 0.1% Triton X-100. One hundred fifty µL of 10 mM *p*-nitrophenyl phosphate was added to the reaction mixture, and the absorbance at 405 nm was photometrically measured using a microplate reader.

Intestinal immune system modulation activity using Peyer's patch cells Peyer's patch cells from the small intestine of C3H/He mice were suspended in RPMI 1640 medium supplemented with 5% FBS (RPMI 1640-FBS). After 180 µL of the cell suspension (2×10⁶ cells/mL) was cultured with a 20 µL of a sample in a 96-well flat bottom microtiter plate for 5 days at 37°C, 50 µL of the resulting culture supernatant was incubated with a bone marrow cell suspension (2.5×10⁵ cells/mL) from C3H/He mice under a humidified atmosphere of 5% CO₂-95% air (20). After 6 days of culture, 20 µL of CCK-8 solution was added to each well and cells were continuously cultured for 4-6 h, then the intensity was measured using a microplate reader at 450 nm. The intestinal immune system modulation activity was expressed as a relative percentage of bone marrow cell growth to a saline control incubated without a sample.

Statistical analysis All values are reported as a mean±standard deviation (SD) of quadruplicate determinations. All statistical analyses were conducted using the Statistical Package for Social Sciences (Version 12.0, SPSS Inc., Chicago, IL, USA). Differences among samples were evaluated using a one-way analysis of variance (ANOVA) and Duncan's multiple range test at *p*<0.05.

Results and Discussion

Immunomodulation enhancement of *H. erinaceum*-fermented ginseng For enhancement of the immunomodulation activity of ginseng, Korean fresh ginseng was fermented with *H. erinaceum* mushroom mycelia in a solid-state culture (SSC) and lyophilized into

fermented ginseng (FG-HE). After hot-water extracts (FG-HE-HW, 39.7% (w/w) yield of FG-HE) were prepared from FG-HE using decoction, FG-HE-HW was fractionated via ethanol precipitation to obtain a crude polysaccharide fraction (FG-HE-CP, 3.23% yield of FG-HE). Hot-water extracts (NG-HW and HE-HW) and the crude polysaccharide (NG-CP and HE-CP) were also fractionated from non-fermented ginseng and *H. erinaceum* mycelia alone as controls.

The mitogenic activity of splenocytes was significantly ($p < 0.05$) higher in FG-HE-HW (1.30x of a saline control at 100 $\mu\text{g/mL}$) than in NG-HW (1.05x) and HE-HW (1.15x) (Fig. 1A). The crude polysaccharide (FG-HE-CP, 1.52x) fractionated from FG-HE-HW also showed a significantly ($p < 0.05$) higher potent mitogenic activity, compared with NG-CP (1.35x) and HE-CP (1.26x) (Fig. 1A). Hot-water extracts of non-fermented and fermented ginseng (NG-HW and FG-HE-HW, respectively) resulted in 1.33x and 1.48x higher macrophage stimulation levels based on lysosomal enzyme activity at 100 $\mu\text{g/mL}$ than saline controls (Fig. 1B). FG-HE-HW also exhibited a significantly ($p < 0.05$) higher macrophage stimulation activity than hot-water extracts from *H. erinaceum* mycelia alone (HE-HW, 1.30x). FG-HE-CP exhibited a significantly ($p < 0.05$) increased macrophage stimulation activity of 1.63x, compared with NG-CP (1.50x) and HE-CP (1.46x). FG-HE-HW did not exhibit a potent intestinal immune system modulation activity thorough Peyer's patch. However, FG-HE-CP (1.35x) exhibited a significantly ($p < 0.05$) higher intestinal immune system modulation activity than controls (NG-CP at 1.18x and HE-CP at 1.27x) (Fig. 1C). Consequently, the crude polysaccharide fraction from fermented ginseng exhibited more enhanced immunomodulation activities than non-fermented fresh ginseng and *H. erinaceum* mycelia alone. Recently, several studies have reported on the results about industrial usefulness of SSC process using mushroom mycelia. Martins *et al.* (21) suggested that solid-state fermentation can produce more bioactive phenolic compounds, which can be applied to food or pharmaceutical, from natural sources than liquid fermentation. SSC can also be high-value substance having the enhanced biological activity from inexpensive agricultural products or waste materials with effective management cost resulting from less energy and water input, the reduced control of fermentation condition and downstream process (22,23). Because of these availabilities, SSC process, which can produce the bioactive compounds more efficiently with higher yields than with submerged fermentation, is an increasing trend on research and industrial application (22).

The roots of *Panax ginseng* C.A. Meyer (Korean ginseng) is the representative herbal medicines that have been administered and studied in chronic diseases treatment as a tonic for a long time in East Asia, such as Korea, China, and Japan. Korean ginseng has been generally distributed to two types in the market. White ginseng (WG) is the dried products after peeling of the wild ginseng, and red ginseng (RG) is steamed and dried from wild ginseng without peeling. Thermal treatment process of ginseng, such as heat or steaming, has been reported to promote the absorption of ginseng nutrition in the small intestine (3), and to increase the physiological

Fig. 1. Immunostimulation activity of hot-water extracts and the crude polysaccharide fraction from *Hericium erinaceum*-fermented ginseng. (A) Mitogenic activity, (B) Macrophage stimulating activity, and (C) Intestinal immune system modulation activity. ¹NG; non-fermented ginseng, HE; *H. erinaceum* mycelia, FG-HE; *H. erinaceum*-fermented ginseng. ²Results (mean $\pm$ SD, $n=4$) with different small letters (a-e) were significant ($p < 0.05$) in each activity.

activity due to structural change of active ingredient (24). Despite these usefulness, Du *et al.* (25) reported that heat process of ginseng could degrade thermally unstable malonyl-ginsenosides, and decrease the level of the essential amino acid (especially Lys) via the Maillard

Table 1. Component content of the immunostimulating polysaccharide fraction from *Hericium erinaceum*-fermented ginseng using solid-state culture

Fraction ¹⁾	Content (%)		
	Neutral sugar	Uronic acid	Protein
NG-CP	85.15±4.71 ^{bc2)}	12.42±2.92 ^b	2.43±1.10 ^a
NG-CP-II	82.40±4.67 ^{bc}	9.65±2.43 ^{ab}	7.95±1.91 ^b
NG-CP-III	65.50±5.37 ^a	26.01±2.71 ^c	8.49±1.51 ^b
HE-CP	86.74±5.29 ^c	6.07±1.12 ^a	7.19±0.76 ^b
HE-CP-II	80.58±4.57 ^{bc}	11.96±1.92 ^b	7.46±0.92 ^b
HE-CP-III	64.82±4.84 ^a	23.55±4.17 ^c	11.63±4.37 ^b
FG-HE-CP	79.67±3.72 ^{bc}	12.89±2.09 ^b	7.44±1.71 ^b
FG-HE-CP-II	78.81±4.31 ^b	10.42±3.29 ^{ab}	10.77±3.38 ^b
FG-HE-CP-III	66.73±4.61 ^a	22.85±3.83 ^c	10.42±3.61 ^b

¹⁾NG-CP, crude polysaccharide from non-fermented ginseng; NG-CP-II/III, polysaccharide fractions eluted from NG-CP at 0.1/0.2 M NaCl on DEAE-Sepharose CL-6B, HE-CP and HE-CP-II/III from *H. erinaceum* mycelia; FG-HE-CP and FG-HE-CP-II/III from *H. erinaceum*-fermented ginseng.

²⁾Results (mean±SD, *n*=4) with different superscripts (^{a-c}) were significant (*p*<0.05) in each component.

reaction. Thus, this study intended to enhance the immunomodulation activity of fresh ginseng using SSC with mycelia instead of heating.

In this study, SSC with ginseng and *H. erinaceum* mycelia enhanced the immunological activity of fresh ginseng (Fig. 1), and component contents of fermented ginseng were different from components of non-fermented ginseng (Table 1). Thus, SSC can replace industrial heating for enhancement of the physiological activities of ginseng.

Since the roots of *P. ginseng* C.A. Meyer have a variety of important physiological activities, ginseng has been widely applied and investigated on prevention and treatment of common illness or chronic diseases as well as homeostasis maintenance. Until recently, the active components involved in a variety of pharmacological activity of ginseng had been mainly studied on ginsenosides (26,27). However, only ginsenosides does not play an important role for all biological activities of ginseng because ginseng contains a large amounts of polysaccharides, peptides and essential oils in addition to ginsenosides (28,29). Among the components other than ginsenosides, ginseng polysaccharides is increasingly getting more attention on immunomodulation, anticancer, and antioxidant (30,31). Therefore, this study was also focused to provide information about immunostimulating effects and chemical properties of polysaccharides from fermented ginseng to help development of functional food materials.

Isolation and identification of an immunostimulating polysaccharide from FG-HE-CP A crude polysaccharide fractionated from hot-water extracts using ethanol precipitation consisted of polysaccharide and proteoglycan macromolecules (32). Neutral sugar, uronic acid, and protein contents of the crude polysaccharide were analyzed for elucidation of differences in component contents between the more active crude polysaccharide from fermented ginseng and from non-

fermented ginseng. NG-CP and HE-CP mainly contained 85.1 and 86.7% neutral sugar and 12.4 and 6.1% uronic acid, respectively, with 2.4 and 7.2% protein. FG-HE-CP was comprised of approximately 79.7% neutral sugar, 12.9% uronic acid, and 7.4% protein (Table 1). The neutral sugar level in FG-HE-CP from fermented ginseng was lower than from NG-CP and HE-CP, and FG-HE-CP exhibited relatively high uronic acid and protein contents. In particular, changes in the main component contents of neutral sugar and uronic acid in the polysaccharide indicated that fresh ginseng was altered during solid-state culture with *H. erinaceum* mycelia.

NG-CP and HE-CP as controls were fractionated into 2 non-adsorbed fractions (NG-CP-I and HE-CP-I) eluted using distilled water, and adsorbed fractions (NG-CP-II-IV and HE-CP-II-VII, respectively) using the same column (Fig. 2A and 2B). In the meanwhile, FG-HE-CP, which exhibited an enhanced immunomodulation activity, compared with non-fermented ginseng, was also further fractionated using anion-exchange chromatography on a DEAE-Sepharose CL-6B (Cl⁻ form) column, and a non-adsorbed (FG-HE-CP-I) and 6 adsorbed fractions (FG-HE-CP-II to VII) were obtained (Fig. 2C). Comparison of elution profiles of crude polysaccharides showed that fractions from FG-HE-CP contained more neutral sugars and less uronic acid than fractions from NG-CP, and that fractions FG-HE-CP-V, VI and VII eluted using NaCl concentrations over 0.4 M were only detected in fractionation of FG-HE-CP (Fig. 2).

FG-HE-CP-III showed the most potent mitogenic activity at 1.95x of a saline control at 100 µg/mL, and a significantly (*p*<0.05) higher mitogenic effect than fractions from non-fermented ginseng and mycelia (NG-CP-III, 1.66x and HE-CP-III, 1.70x, respectively) (Table 2). Fractions from the crude polysaccharide on a DEAE-Sepharose CL-6B column were investigated for macrophage stimulation activity *in vitro*. FG-HE-CP-II and III exhibited potent activities (2.01 and 1.96x, respectively) compared with the same NaCl elution fractions from non-fermented ginseng (1.40-1.72x) and *H. erinaceum* mycelia alone (1.51-1.75x) (Table 2).

Fractions from the crude polysaccharide of fermented ginseng were examined for production of functional materials with intestinal immune system modulation activity using Peyer's patch cells. FG-HE-CP-III exhibited significantly (*p*<0.05) higher activity levels (1.73x) than any fraction from controls (NG-CP-II and III, 1.41 and 1.20x; HE-CP-II and III, 1.36 and 1.32x, respectively) (Table 2). Thus, FG-HE-CP-III exhibited potent activities in all assays of immunological activity (Table 2) and the highest yield (17.56% of FG-HE-CP) among carbohydrate-rich fractions.

The active carbohydrate-rich fractions FG-HE-CP-II and III were comprised of 78.8 and 66.7% neutral sugar, respectively, and 10.4 and 22.9% uronic acid (Table 1). The less active polysaccharides from non-fermented ginseng (NG-CP-II/III) and *H. erinaceum* mycelia alone (HE-CP-II/III) also contained 82.4/65.5% and 80.6/64.8% neutral sugar with 9.65/26.0% and 12.0/23.6% uronic acid (Table 1). Fractionation using a DEAE-Sepharose CL-6B column resulted in similar component contents of neutral sugars and uronic acid of

Fig. 2. Elution profile of the crude polysaccharide fraction from *Hericium erinaceum*-fermented ginseng on DEAE-Sepharose CL-6B column chromatography. (A) Fractionation of NG-CP, (B) Fractionation of HE-CP, and (C) Fractionation of FG-HE-CP. Column size; 4.0×40 cm.

active polysaccharide fractions from FG-HE-CP, NG-CP, and HE-CP, probably due to elution at the same ionic strength. Therefore, component sugars were analyzed for identification of compositional differences of these active fractions from FG-HE-CP and FG-CP or HE-CP. NG-CP-III, HE-CP-III, and FG-HE-CP-III appeared to be acidic polysaccharides (33) containing 29.31, 24.75, and 27.47 mole% of uronic acidic (sum of GalA and GlcA) sugars (Table 3). NG-CP-III also contained 27.79 and 23.26 mole% of the main neutral sugars Ara and Gal, respectively, and 7.60 mole% Rha and 5.28 mole% Glc as minor components.

Table 2. Immunostimulation activity of polysaccharide subfraction from the crude polysaccharide of *Hericium erinaceum*-fermented ginseng using DEAE-Sepharose CL-6B column chromatography

Fraction ¹⁾ (100 mg/mL)	Relative activity (%)		
	Mitogen ²⁾	Macrophage	Intestinal immune
Saline control	100.0±4.5 ^{a3)}	100.0±9.2 ^a	100.0±3.8 ^a
NG-CP	135.3±9.8 ^{bc}	150.2±5.9 ^{bc}	118.1±3.9 ^{bc}
NG-CP-II	140.4±3.7 ^{bc}	157.0±35.2 ^{bc}	140.9±7.4 ^e
NG-CP-III	166.3±9.1 ^d	171.6±10.6 ^{cd}	120.3±9.4 ^{bc}
NG-CP-IV	152.5±16.4 ^{cd}	139.7±12.2 ^b	109.9±2.7 ^{ab}
HE-CP	125.8±17.0 ^b	145.7±12.4 ^b	127.1±9.8 ^{cd}
HE-CP-II	150.5±15.7 ^{cd}	159.0±13.7 ^{bc}	135.6±4.5 ^{de}
HE-CP-III	169.5±5.7 ^d	175.3±10.9 ^{cde}	132.4±3.8 ^{de}
HE-CP-IV	142.3±15.6 ^{bc}	150.9±12.7 ^{bc}	124.8±11.7 ^{cd}
FG-HE-CP	151.9±21.3 ^{cd}	163.4±9.9 ^{bc}	135.4±6.1 ^{de}
FG-HE-CP-II	165.5±10.7 ^d	200.6±20.4 ^f	157.8±2.9 ^f
FG-HE-CP-III	194.8±15.4 ^e	195.9±16.4 ^{ef}	172.7±5.2 ^g
FG-HE-CP-IV	149.2±10.3 ^{cd}	188.0±7.8 ^{def}	139.6±11.8 ^e

¹⁾NG-CP, crude polysaccharide from non-fermented ginseng; NG-CP-II/III/IV, polysaccharide fraction from NG-CP; HE-CP and HE-CP-II/III/IV from *H. erinaceum* mycelia; FG-HE-CP and FG-HE-CP-II/III/IV from *H. erinaceum*-fermented ginseng.

²⁾Mitogen, mitogenic activity of splenocytes; macrophages, macrophage stimulating activity of the lysosomal enzyme; and intestinal immune, intestinal immune system modulating activity through Peyer's patch.

³⁾Results (mean±SD, n=4) with different superscripts (^{a-g}) were significant ($p<0.05$) in each activity.

Table 3. Component sugar analysis of immunostimulating polysaccharide fraction from the crude polysaccharide of *Hericium erinaceum*-fermented ginseng

Component sugar (mole%)	NG-CP ¹⁾		HE-CP		FG-HE-CP	
	II	III	II	III	II	III
Ara	22.62	27.79	0.11	2.22	5.68	11.81
Xyl	1.55	2.38	0.06	4.68	4.87	4.40
Rha	3.64	7.60	0.34	3.58	0.19	5.55
Fuc	2.47	1.78	8.97	5.44	4.69	3.02
Man	6.44	2.61	15.98	11.13	12.25	8.25
Gal	40.78	23.26	30.34	21.32	35.21	26.15
Glc	14.27	5.28	28.69	26.88	24.92	13.35
GalA	5.44	22.04	10.00	15.93	7.65	19.21
GlcA	2.79	7.27	5.51	8.82	4.54	8.26

¹⁾NG-CP-II/III, polysaccharide fractions from crude polysaccharide of non-fermented ginseng; HE-CP-II/III, from *H. erinaceum* mycelia; FG-HE-CP-II/III, from *H. erinaceum*-fermented ginseng.

FG-HE-CP-III contained similar 26.15 and 27.47 mole% proportions of Gal and uronic acid as NG-CP-III. The 11.81 mole% of Ara was less than that of NG-CP-III, but the 8.25 mole% of Man was higher than for NG-CP-III (Table 3). Thus, the active polysaccharide from fermented ginseng was probably modified by *H. erinaceum* mycelial growth, or by an enzyme released from mycelia. The active medium polysaccharide fractions NG-CP-II and FG-HE-CP-II contained 40.78 and 35.21 mole% Gal, respectively, 14.27 and 24.92 mole% Glc, 22.62 and 5.68 mole% Ara, and 6.44 and 12.25 mole% Man, in

Fig. 3. Effects of chemical and enzymatic treatments of the active polysaccharide fraction (FG-HE-CP-III) from *Hericium erinaceum*-fermented ginseng on immunostimulation activity. ¹Mitogen; mitogenic activity of splenocytes, macrophages; macrophage stimulating activity of the lysosomal enzyme, and intestinal immune; intestinal immune system modulation activity through Peyer's patch. ²Results (mean±SD, n=4) with different small letters (a-d) were significant ($p < 0.05$) in each activity.

addition to a small amount of uronic acid (Table 3). HE-CP-II and III consisted mainly of Gal, Glc, Man, and uronic acid at molar ratios of 1.00:0.95:0.53:0.51 and 0.79:1.00:0.41:0.92, respectively, which were different from compositions of FG-HE-CP-II and III. In addition, when the FG-HE-CP-III active fraction was treated with NaIO₄ and pronase in order to elucidate which moiety contributed to the activity, NaIO₄ oxidation significantly ($p < 0.05$) decreased the activity of FG-HE-CP-III by 51.0–76.9%, whereas pronase digestion did not affect the activity (Fig. 3).

Oxidation of carbohydrates by the periodate ion was, for a long time, a classical method used for structural determination of complex carbohydrates. NaIO₄ oxidation of polysaccharides can oxidize vicinal hydroxyl groups and affect polysaccharide structure (16). Thus, the neutral polysaccharide moiety in FG-HE-CP-III can contribute to expression of the enhanced immunological activity of ginseng.

Eighteen pharmacologically active polysaccharides from ginseng roots were identified, consisting mainly of neutral sugars containing Ara, Gal, Rha, and Glc, and uronic acid with different molar ratios (34,35). Structural analysis indicated that ginseng polysaccharides were arabinogalactan, starch-like glucan and acidic pectic polysaccharides with Mw=3,500–160,000 da. Component sugar analysis showed that the polysaccharide from nonfermented ginseng was similar to NG-CP-II or III (Table 3). Although the active FG-HE-CP-III polysaccharide from fermented ginseng contained amounts of Gal and uronic acid similar to non-fermented ginseng, the compositional difference of Ara and Man between FG-HE-CP-III and NG-CP-III (Table 3) indicated that SSC with mycelia had an effect on the ginseng composition.

This study did not specifically identify whether changes of

component sugars and linkages of polysaccharides in fermented ginseng resulted from mycelial attachment due to mycelium growth, or biotransformation by mycelia or a mycelial enzyme for fermentation. However, SSC with ginseng and mycelia produced enhancement of the immunomodulation activity.

Acknowledgment This research was supported by a grant from the Academic Research Program of Korea National University of Transportation in 2014.

Disclosure The authors declare no conflict of interest.

References

- Lee HS, Lee HJ, Yu HJ, Ju DW, Kim Y, Kim CT, Kim CJ, Cho YJ, Kim N, Choi SY, Suh HJ. A comparison between high hydrostatic pressure extraction and heat extraction of ginsenosides from ginseng (*Panax ginseng* C.A. Meyer). *J. Sci. Food Agr.* 91: 1466–1473 (2011)
- Kim WY, Kim JM, Han SB, Lee SK, Kim ND, Park MK, Kim CK, Park JH. Steaming of ginseng at high temperature enhances biological activity. *J. Nat. Prod.* 63: 1702–1704 (2000)
- Nam KY. The comparative understanding between red ginseng and white ginsengs, processed ginsengs (*Panax ginseng* C.A. Meyer). *J. Ginseng Res.* 29: 1–18 (2005)
- Hwang IG, Kim HY, Jeong EM, Woo KS, Jeong JH, Yu KW, Lee J, Jeong HS. Changes in ginsenosides and antioxidant activity of Korean ginseng (*Panax ginseng* C.A. Meyer) with heating temperature and pressure. *Food Sci. Biotechnol.* 19: 941–949 (2010)
- Kwon SW, Han SB, Park IH, Kim JM, Park MK, Park JH. Liquid chromatographic determination of less polar ginsenosides in processed ginseng. *J. Chromatogr. A* 921: 335–339 (2001)
- Park IH, Han SB, Kim JM, Piao L, Kwon SW, Kim NY, Kang TL, Park MK, Park JH. Four new acetylated ginsenosides from processed ginseng (sun ginseng). *Arch. Pharm. Res.* 25: 837–841 (2002)
- Lee JH, Shim JS, Lee JS, Kim MK, Chung MS, Kim KH. Pectin-like acidic polysaccharide from *Panax ginseng* with selective antiadhesive activity against pathogenic bacteria. *Carbohydr. Res.* 341: 1154–1163 (2006)
- Luo D, Fang B. Structural identification of ginseng polysaccharides and testing of their antioxidant activities. *Carbohydr. Polym.* 72: 376–381 (2008)
- Pandey A, Soccol CR, Mitchell D. New developments in solid state fermentation: I-bioprocesses and products. *Process Biochem.* 35: 1153–1169 (2000)
- Park CK, Kim H, Tu Q, Yu KW, Jeong HS, Lee HY, Jeong JH. Chemical composition and immunomodulating activity of the fermented Korean ginseng (*Panax ginseng* C.A. Meyer) with mushroom mycelium by solid culture. *J. Korean Soc. Food Sci. Nutr.* 38: 1145–1152 (2009)
- Dubois M, Gilles KA, Hamilton JK, Rebers RA, Smith F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28: 350–356 (1956)
- Blumenkrantz N, Asboe-Hansen, G. New method for quantitative determination of uronic acid. *Anal. Biochem.* 54: 484–489 (1973)
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254 (1976)
- Jones TM, Albersheim P. A gas chromatographic method for the determination of aldose and uronic acid constituents of plant cell wall polysaccharide. *Plant Physiol.* 49: 926–936 (1972)
- Zhao JF, Kiyohara H, Yamada H, Takemoto N, Kawamura H. Heterogeneity and characterization of mitogenic and anti-complementary pectic polysaccharides from the roots of *Glycyrrhiza uralensis* Fisch et DC. *Carbohydr. Res.* 219: 149–172 (1991)
- Yamada H, Kiyohara H, Cyong JC, Takemoto N, Komatsu Y, Kawamura H, Aburada M, Hosoya E. Fractionation and characterization of mitogenic and anti-complementary active fraction from Kampo (Japanese herbal) medicine 'Juzen-taiho-to'. *Planta Med.* 56: 386–391 (1990)
- Ishiyama M, Tominaga H, Shiga M, Sasamoto K, Ohkura Y, Ueno K. A combined assay of cell viability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. *Biol. Pharm. Bull.* 19: 1518–1520 (1996)
- Conrad RE. Induction and collection of peritoneal exudates macrophages. pp.

- 5-11. In: Manual of Macrophage Methodology. Herscowitz BH, Holden HT, Bellanti JA, Ghaffar A (eds). Marcel Dekker Inc., New York, NY, USA (1981)
19. Suzuki I, Tanaka H, Kinoshita A, Oikawa S, Osawa M, Yadomae T. Effect of orally administered-glucan on macrophage function in mice. *Int. J. Immunopharmacol.* 12: 675-684 (1990)
20. Yu KW, Kiyohara H, Matsumoto T, Yang HC, Yamada H. Intestinal immune system modulating polysaccharides from rhizomes of *Atractylodes lancea* DC. *Planta Med.* 64: 714-719 (1998)
21. Martins S, Mussatto SI, Martínez-Avila G, Montañez-Saenz J, Aguilar CN, Teixeira JA. Bioactive phenolic compounds: Production and extraction by solid-state fermentation. A review. *Biotechnol. Adv.* 29: 365-373 (2011)
22. Hölker U, Lenz J. Solid-state fermentation: Are there any biotechnological advantages? *Curr. Opin. Microbiol.* 8: 301-306 (2005)
23. Nigam PS. Production of bioactive secondary metabolites. pp. 129-145. In: *Biotechnology for Agro-industrial Residues Utilization*. Nigam PS, Pandey A (eds). Springer, Berlin, Germany (2009)
24. Baek NI, Kim DS, Lee YH, Park JD, Lee CB, Kim SI. Ginsenoside Rh4, a genuine dammarane glycoside from Korean red ginseng. *Planta Med.* 62: 86-87 (1996)
25. Du XW, Wills RBH, Stuart DL. Changes in neutral and malonyl ginsenosides in American ginseng (*Panax quinquefolium*) during drying, storage and ethanolic extraction. *Food Chem.* 86: 155-159 (2004)
26. Christensen LP. Ginsenosides chemistry, biosynthesis, analysis, and potential health effects. *Adv. Food Nutr. Res.* 55: 1-99 (2009)
27. Yoon SR, Lee GD, Park JH, Lee IS, Kwon JH. Ginsenoside composition and antiproliferative activities of explosively puffed ginseng (*Panax ginseng* C.A. Meyer). *J. Food Sci.* 75: C378-C382 (2010)
28. Attele AS, Wu JA, Yuan CS. Ginseng pharmacology: Multiple constituents and multiple action. *Biochem. Pharmacol.* 58: 1685-1693 (1999)
29. Choi KT. Botanical characteristics, pharmacological effects and medicinal components of Korean *Panax ginseng* C.A. Meyer. *Acta Pharmacol. Sin.* 29: 1109-1118 (2008)
30. Sun Y. Structure and biological activities of the polysaccharides from the leaves, roots and fruits of *Panax ginseng* C.A. Meyer: An overview. *Carbohydr. Polym.* 85: 490-499 (2011)
31. Wang J, Li S, Fan Y, Chen Y, Liu D, Cheng H, Gao X, Zhou Y. Anti-fatigue activity of the water-soluble polysaccharides isolated from *Panax ginseng* C. A. Meyer. *J. Ethnopharmacol.* 130: 421-423 (2010)
32. Yu KW, Kim YS, Shin KS, Kim JM, Suh HJ. Macrophage-stimulating activity of exo-biopolymer from cultured rice bran with *Monascus pilosus*. *Appl. Biochem. Biotech.* 126: 35-48 (2005)
33. Rana V, Kumar V, Soni PL. Structural characterization of an acidic polysaccharide from *Dalbergia sissoo* Roxb. leaves. *Carbohydr. Polym.* 90: 243-250 (2012)
34. Gao QP, Kiyohara H, Cyong JC, Yamada H. Chemical properties and anti-complementary activities of polysaccharide fractions from roots and leaves of *Panax ginseng*. *Planta Med.* 55: 9-12 (1989)
35. Tomoda M, Hirabayashi K, Shimizu N, Gonda R, Ohara N, Takada K. Characterization of two novel polysaccharides having immunological activities from the root of *Panax ginseng*. *Biol. Pharm. Bull.* 16: 1087-1090 (1993)