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Characterization of an Immune-Enhancing Polysaccharide Fraction Isolated from Heat-Processed Ginseng Derived from *Panax ginseng* C.A. Meyer

Sung Jin Kim [1,†], Myoung-Sook Shin [1,†], Minyeong Kim [2], Seung-Hoon Baek [2,*] and Ki Sung Kang [1,*]

[1] College of Korean Medicine, Gachon University, Seongnam 13120, Korea; sungjinkim001@gmail.com (S.J.K.); ms.shin@gachon.ac.kr (M.-S.S.)
[2] College of Pharmacy and Research Institute of Pharmaceutical Science and Technology (RIPST), Ajou University, Suwon 16499, Korea; my1123@ajou.ac.kr
* Correspondence: shbaek@ajou.ac.kr (S.-H.B.); kkang@gachon.ac.kr (K.S.K.); Tel.: +82-31-219-3449 (S.-H.B.); +82-31-750-5402 (K.S.K.)
† These authors contributed equally to this work.

Abstract: *Panax ginseng* C.A. Meyer (ginseng) has shown immune-enhancing activity in many studies. The purpose of the present study was to analyze the chemical properties of a polysaccharide fraction (SGP) purified from heat- processed ginseng and to evaluate its immune-enhancing activity using RAW264.7 macrophages. The results showed that SGP increased inducible nitric oxide synthase expression and nitric oxide production in RAW264.7 macrophages. In addition, SGP increased mRNA expression and secretion of interleukin 6 and tumor necrosis factor alpha. Immunoblotting results showed that SGP increased the phosphorylation of mitogen-activated protein kinases (MAPKs) and NF-κB subunit p65 at 500 μg/mL and 1000 μg/mL. Taken together, SGP can activate macrophages through the MAPK and NF-κB signaling pathways, and it may help maintain homeostasis during viral and bacterial infections.

Keywords: *Panax ginseng* C.A. Meyer; heat-processed; polysaccharide; innate immune enhancing

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1. Introduction

Macrophages are responsible for innate immune responses to foreign organisms or pathogenic infections by regulating effector cells such as B and T lymphocytes [1,2]. Activated macrophages immediately remove pathogens through phagocytosis and nitric oxide (NO) production [3,4]. Pathogens internalized through phagocytosis are subsequently expressed as complexes on the surface of macrophages, along with major histocompatibility complexes, through the antigen presentation process [5,6]. In addition, macrophages secrete NO and cytokines (interleukin [IL]-1, IL-6, and tumor necrosis factor alpha [TNF-α]) to activate adaptive immunity and promote differentiation of B cells into plasma cells [7,8]. Cytokines are produced by the cell surface receptor pattern recognition receptor (PRR) [9,10]. Immune receptors can be activated by cytokine production through PRRs such as Toll-like receptors, Dectin-1, mannose receptors, and complement receptors. These PRRs regulate the secretion of cytokines such as TNF-α and IL-6 in macrophages through mitogen-activated protein kinases (MAPKs) and NF-κB [11,12].

Panax ginseng C.A. Meyer (here termed ginseng) has been used for thousands of years to treat diseases and to help maintain the body's homeostasis [13,14]. The active components of ginseng are ginsenosides, polysaccharides, and polypeptides, and their pharmacological activities including anti-fatigue, anti-cancer, and immune enhancement effects have been previously reported [15,16]. Ginseng products are referred to by various terms, depending on the processing method: freshly harvested ginseng is termed fresh ginseng, dried ginseng is referred to as white ginseng, and steamed dried ginseng is termed red ginseng [17,18]. Through such processing, the biological activity of ginseng can be

improved by altering the content of ginsenosides, such as a decrease in ginsenosides Rg1, Rb1, Rc, and Rd and an increase in ginsenosides Rg2, Rg3, and Rh1.

Recently, it has been reported that natural product-derived polysaccharides can induce innate immune-enhancing activities such as those of macrophages, NK cells, and bone marrow cells, as mediated by Peyer's patches [19–24]. Therefore, in this study, we isolated a polysaccharide fraction (termed SGP) through a new processing method, the product of which is termed heat-processed ginseng and analyzed its chemical properties. We then evaluated macrophage-stimulatory activities of SGP and identified effects on intercellular signaling pathways using RAW264.7 macrophages.

2. Materials and Methods

2.1. Antibodies and Reagents

The phosphorylation specific antibodies of JNK, ERK1/2, p38, and p65 and antibodies of JNK, ERK1/2, p38, p65 (C22B4), β-actin (13E5), and inducible nitric oxide synthase (iNOS; D6B6S) were supplemented with commercial products (CST, Danvers, MA, USA). Secondary antibodies were supplemented by Santa Cruz Biotechnology (Paso Robles, CA, USA). An RNeasy kit was supplemented by Qiagen (Redwood City, CA, USA). Real-time qPCR SYBR green reagent was obtained by Applied Biosystems (Foster City, CA, USA). Mouse interlukin-6 and tumor necrotic factor-α ELISA sets were evaluated by BD Biosciences (San Jose, CA, USA). Ez-Cytox solution was assayed for DoGEN (Seoul, Republic of Korea). Sulfanilamide, phosphoric acid, and naphthyl ethylene-diamine dihydrochloride were analyzed using Sigma-Aldrich (St. Louis, MO, USA). Antibiotics and fetal bovine serum were purchased from Gibco (Grand Island, NY, USA).

2.2. Plant Material and Preparation of SGP

Dried root of four-year-old ginseng was purchased from Nonghyup (Seoul, Korea). A voucher specimen (A16077) was authenticated and deposited by Prof. Hong Pyo Kim, laboratory of pharmacognosy in College of Pharmacy in Ajou University. Heat-processed ginseng was prepared by steaming ginseng at 120 °C for 3 h using an autoclave. Dried heat-processed ginseng (50 g) was used for extracting with distilled water (500 mL) under reflux conditions for 3 h. The extraction was repeated three times, and the extracts were pooled and concentrated to 200 mL using an evaporator. Protein residues were removed from the extract by three treatments with Sevag reagent (CHCl3:BuOH = 4:1, v/v; 200 mL) using liquid–liquid extraction. The resulting aqueous layer was mixed with four volumes of ethanol, and the mixture was stored at 4 °C overnight. After centrifugation (4500× g, 5 min, 4 °C), the precipitate was reconstituted with distilled water and then passed through a column filled with Diaion HP-20 resin (5 × 20 cm) preconditioned with ethanol and water. The aqueous eluent was dialyzed using a cellulose tubular membrane (CelluSep T2, MWCO 6000–8000; Membrane Filtration Products, Sequin, TX, USA). Polysaccharide of heat-processed ginseng (SGP) were prepared by freeze-drying and were then subjected to chemical analysis and biological assays.

2.3. Chemical Characteristic Analysis of SGP

The molecular weight distribution of SGP was evaluated through high-performance gel permeation chromatography (HP-GPC) using a Flexar HPLC system equipped with a reflective index detector (PerkinElmer, Waltham, MA, USA). The PL Aquagel-OH 30 column (100~60,000 Da, 8 μm, 300 × 7.5 mm, PL1120-6830, Agilent, Santa Clara, CA, USA) and PL Aquagel-OH 40 column (10,000~200,000 Da, 8 μm, 300 × 7.5 mm, PL1149-6840, Agilent) were connected to the HP-GPC system. Each solution (1 mg/mL) of standards (Pullulan 47.3–788 kDa; Showa Denko, Tokyo, Japan) and SGP were prepared by dissolving them in distilled water. The injection volume was 30 μL. The eluent was 0.2 M NaCl at a flow rate of 1 mL/min.

The total carbohydrate, uronic acid, and protein contents in SGP were assessed using a phenol-sulfuric acid method [25], an m-hydroxydiphenyl assay [26], and a Bradford

assay (Quick Start™ Bradford Protein Assay; Bio-Rad Hercules, CA, USA), respectively. The reference standards for each assay were glucose, galacturonic acid, and bovine serum albumin, respectively.

The monosaccharide composition of SGP was assessed using 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization and liquid chromatographic analysis following the modified method of Guo et al. [27]. Briefly, 200 µg SGP was hydrolyzed with 100 µL 2.5 M trifluoroacetic acid (TFA) at 120 °C for 90 min in an autoclave. The hydrolysate was dried under a stream of nitrogen to remove excess TFA with the addition of methanol. The dried hydrolysate was dissolved in 100 µL water, followed by addition of 400 µL 35% ammonia solution and 25 µL 0.5 M PMP solution prepared in methanol. The mixture was incubated at 70 °C for 30 min and was then dried under nitrogen. The residue was dissolved in 1 mL water and was washed three times using 1 mL chloroform. The aqueous layer containing the PMP-derivatized monosaccharides was filtered through a 0.45-µm nylon filter, and 1 µL was injected into the UPLC system equipped with a Shim-pack GIST column (2.1 × 100 mm, 2 µm; Shimadzu, Kyoto, Japan). A mixture of eight sugar standards was derivatized in a range of 0.01 to 0.25 nmol/tube and was analyzed in the same way. Mobile phase A was a mixture (10:90, *v*/*v*) of acetonitrile and aqueous solution (0.045% KH2PO4, *w*/*v*; 0.05% triethylamine, *v*/*v*; final pH 7.5 with H3PO4). Mobile phase B was acetonitrile. Isocratic elution of mobile phases A and B (90:10) was used at a flow rate of 0.3 mL/min. Column oven temperature was maintained at 35 °C. PMP-labeled monosaccharides were detected at 245 nm wavelength.

2.4. Cell Culture

RAW264.7 (Korean Cell Line Bank, Seoul, Korea) cells were grown in DMEM with 10% FBS and 1% of penicillin and streptomycin. Cell growth conditions were determined at 37 °C and inside humidified 5% CO_2.

2.5. Cell Cytotoxicity Assay

RAW264.7 macrophages were grown in 96-well flat-bottomed microplates (1.0×10^5 cells/well). SGP was diluted using culture medium (500 and 1000 µg/mL), and the cells were treated for 24 h. Cell proliferation was investigated with Ez-Cytox solution. Briefly, the Ez-Cytox solution was added into each well and reacted at 37 °C for 30 min. Absorbance was observed with a microplate reader at 450 nm.

2.6. Determination of Nitric Oxide (NO) Production

RAW264.7 macrophages were grown in 96-well flat-bottomed microplates (1.0×10^5 cells/well). SGP was diluted using culture medium (500 and 1000 µg/mL), and the cells were treated for 24 h. NO produced in culture supernatant was mixed with Griess reagent and the same volume of culture supernatant, and absorbance was observed with a microplate reader at 550 nm. NO levels in supernatants were measured using a sodium nitrite dilution standard curve [28].

2.7. Determination of Cytokines TNF-α and IL-6

RAW264.7 macrophages were seeded in 96-well flat-bottomed microplates (1.2×10^5 cells/well). The cells were treated with indicated concentration of SGP (500 or 1000 µg/mL) for 20 h. Cytokine amounts in the supernatant were evaluated with an ELISA set following the manufacturer's guidance.

2.8. Real-Time PCR (RT-qPCR)

RAW264.7 macrophages were seeded in 6-well flat-bottomed microplates (2.0×10^6 cells/well). The cells were then treated with SGP (500 or 1000 µg/mL) inserted into the culture medium for 12 h. After stimulating with SGP for 12 h, total RNA isolated and purified using RNeasy Mini Kit (Qiagen, Vlaencia, CA, USA) For cDNA synthesis, RevertAid First Strand cDNA synthesis kit (Fermentas, MA, USA) was analyzed following

the manufacturer's guidance. RT-qPCR was determined from SYBR Green Master Mix (Applied Biosystems) using the following respective sense and anti-sense primers–IL-6: 5′-GAGGATACCACTCCCAACAG-3′ and 5′-AAGTGCATCATCGTTGTTCA-3′; TNF-α: 5′-GCCTCTTCTCATTCCTGCTTG-3′ and 5′-CTGATGAGAGGGAGGCCATT-3′; GAPDH (used as a housekeeping gene): 5′-GAGGATACCACTCCCAACAG-3′ and 5′-AAGTGCATC ATCGTTGTTGTTCA-3′. IL-6 and TNF-α amplification conditions were evaluated by a Quantstudio 3 real-time PCR system (Applied Biosystems). mRNA levels were calculated from the ΔΔCt method [29].

2.9. Immunoblotting

RAW264.7 macrophages were seeded in 6-well flat-bottomed microplates (2.0×10^6 cells/well). SGP was diluted using culture medium (500 and 1000 μg/mL), and the cells were stimulated for 30 min (for MAPKs and NF-κB protein levels) or 22 h (iNOS protein level). After the treatments, cells were purified three times using DPBS, were harvested, and were lysed using cold radioimmunoprecipitation assay buffer (T&I, Chuncheon, Republic of Korea) containing a phosphatase inhibitor cocktail. Proteins were extracted by centrifugation, and protein analysis was performed on each supernatant. Proteins were separated from the mini-gel 12% SDS-PAGE and transferred to the PVDF membrane, which was blocked using 5% skim milk at room temperature for 2 h. Then PVDF membranes were incubated with primary antibodies for overnight, and after that the membranes were attached to the anti-rabbit secondary antibody linked with an HRP. The protein levels were detected using ECL (Thermo Fisher Scientific) reagent, and development was determined using a Fusion Solo Chemiluminescence System (Vilber Lourmat, Paris, France) ECL detection system.

2.10. Statistical Analysis

All analysis was expressed as means ± SD of triplicate experiments. Statistical analysis was calculated from a one-way ANOVA followed by Tukey's post-hoc test using Prism 8 (GraphPad Software, San Diego, CA, USA). Differences were reported to be statistically significant at $p < 0.05$, $p < 0.001$, and $p < 0.0001$.

3. Results

3.1. Extraction and Chemical Characteristics of SGP

The extraction and fractionation procedure for preparing SGP is shown in Figure 1A. Sevag reagent and Diaion HP-20 resin were used to enrich carbohydrates and to avoid contamination with other plant-derived impurities such as proteins and phenolic compounds [30]. SGP yield was 25% (*w*/*w*). To examine whether SGP was a macromolecular extract of polysaccharides, its molecular weight distribution was assessed using HP-GPC and pullulan molecular weight markers (Figure 1B). SGP showed a broad molecular weight distribution ranging from 5.9 to 788 kDa. Two distinct peaks occurred at 12.6 and 14.2 min retention time. The respective molecular weights were 245.7 and 67.6 kDa. These results imply that SGP is a macromolecular of heat-processed ginseng extract.

To examine whether SGP was a polysaccharide-rich fraction, we first determined its chemical composition using colorimetric assays. As shown in Figure 2A and Table 1, total carbohydrate, uronic acid, and protein levels (%, *w*/*w*) in SGP were 84.3% ± 2.1%, 5.5% ± 0.6%, and 1.50% ± 0.02%, respectively. These data suggest that SGP was mainly composed of carbohydrates. The monosaccharide composition of SGP was determined using PMP-derivatization and HPLC analysis. Component monosaccharides were liberated from polymers by hydrolysis of glycosidic linkages. PMP-derivatized monosaccharides were analyzed using reverse-phase HPLC and a UV detector. Representative chromatograms are shown in Figure 2B and Table 1. The content (%, *w*/*w*) of monosaccharides was determined using standard calibration curves. Glucose (67.3%) was the most dominant monosaccharide in SGP, followed by fucose (4.4%), arabinose (3.2%), galactose (2.8%), galacturonic acid

(1.8%), and trace levels of mannose, rhamnose, and glucuronic acid. These results suggest that SGP is a polysaccharide extract of heat-processed ginseng.

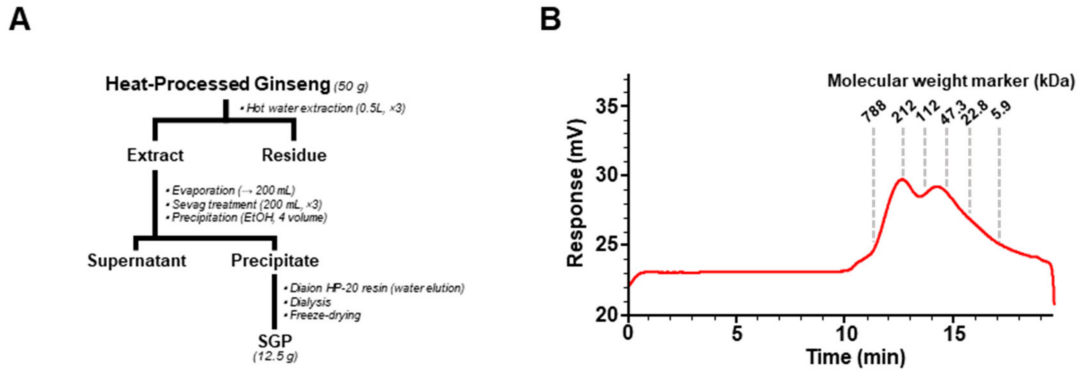

Figure 1. Preparation and molecular weight distribution of heat-processed ginseng polysaccharide fraction (SGP)**.** (**A**) Extraction and fractionation scheme for preparation of SGP. (**B**) HP-GPC chromatogram of SGP. Dotted lines indicate the elution time of each molecular weight marker.

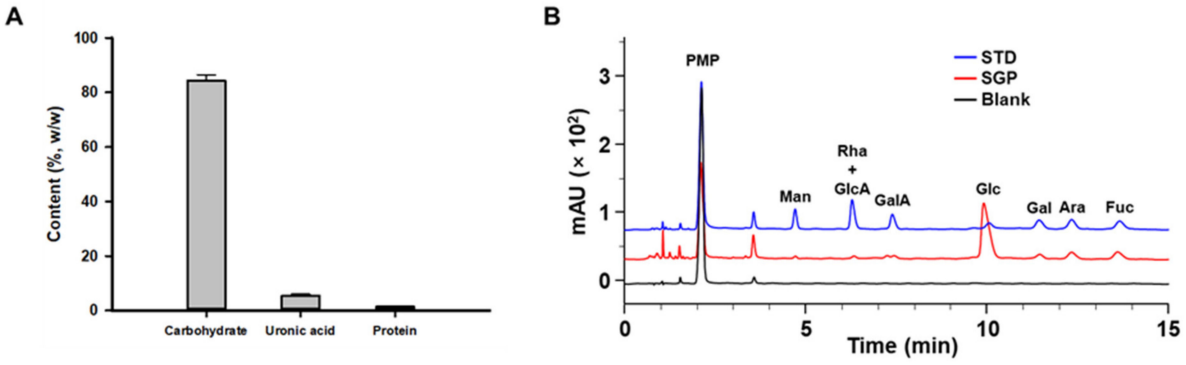

Figure 2. Chemical properties and monosaccharide composition of SPG. (**A**) Total carbohydrate, uronic acid, and protein levels in SGP. Bars indicate means ± standard deviation. (**B**) Overlaid chromatogram of PMP-labeled standard and SGP. (Ara, arabinose; Fuc, fucose; Gal, galactose; GalA, galacturonic acid; Glc, glucose; GlcA, glucuronic acid; Man, mannose; Rha, rhamnose).

Table 1. Chemical properties of SGP isolated from heat-processed ginseng.

Chemical Characteristic (%)	SGP
Carbohydrate	84.3 ± 2.1
Uronic acid	5.5 ± 0.6
Protein	1.5 ± 0.02
Component of Monosaccharides (%)	
Arabinose	3.2
Fucose	4.4
Galactose	2.8
Galacturonic acid	1.8
Glucose	67.3
Glucuronic acid	TL
Mannose	TL
Rhamnose	TL

TL, Trace level.

3.2. Effect of SGP on RAW264.7 Macrophage Cell Proliferation

To investigate the effect of SGP on macrophage proliferation in RAW264.7 macrophages, we first evaluated cell viability. RAW264.7 macrophages were inserted into SGP at 500 and 1000 μg/mL for 22 h. The results showed that treatment with 500 and 1000 μg/mL SGP was non-cytotoxic and slightly increased cell proliferation (Figure 3). Therefore, 500 and 1000 μg/mL SGP were used in subsequent experiments.

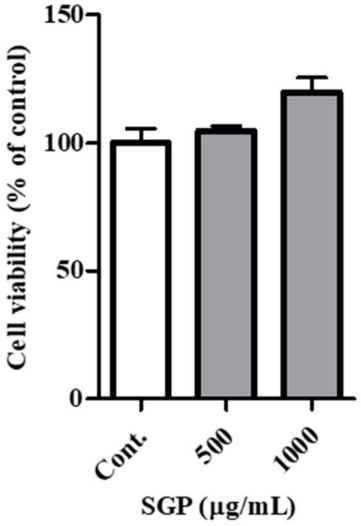

Figure 3. Effect of SGP on the proliferation of RAW264.7 macrophages. RAW264.7 cells were stimulated with indicated concentration of SGP for 22 h, and cell proliferation was evaluated an EZ-Cytox reagent.

3.3. SGP Increased Immune-Enhancing Activity through NO and iNOS Level in RAW264.7 Macrophages

Polysaccharides isolated from ginseng were reported to exert immune-enhancing effects [31,32]. iNOS is an important factor to produce NO and activate macrophages. In this study, we investigated NO and iNOS levels in SGP-treated RAW264.7 macrophages. RAW264.7 macrophages were stimulated with 500 or 1000 μg/mL SGP for 22 h (for NO) or 18 h (for iNOS). The results showed that treatment with 500 and 1000 μg/mL SGP slightly increased NO (Figure 4A) and iNOS levels. As shown in the quantification graph of iNOS intensity, SGP increased iNOS level in RAW264.7 macrophages (Figure 4B).

3.4. SGP Increased mRNA Expression and Cytokine Secretion of IL-6 and TNF-α in RAW264.7 Macrophages

To evaluate the effect of SGP on the secretion of IL-6 and TNF- α in RAW264.7 macrophages, ELISA and RT-qPCR analysis were performed. The results indicated that secretion of IL-6 and TNF-α levels increased in RAW264.7 cells after treatment with SGP at 500 and 1000 μg/mL (Figure 5A,B). In addition, we investigated IL-6 and TNF-α mRNA levels in SGP in RAW264.7 cells, both of which increased at SGP concentrations of 500 and 1000 μg/mL in RAW264.7 macrophages (Figure 5C,D).

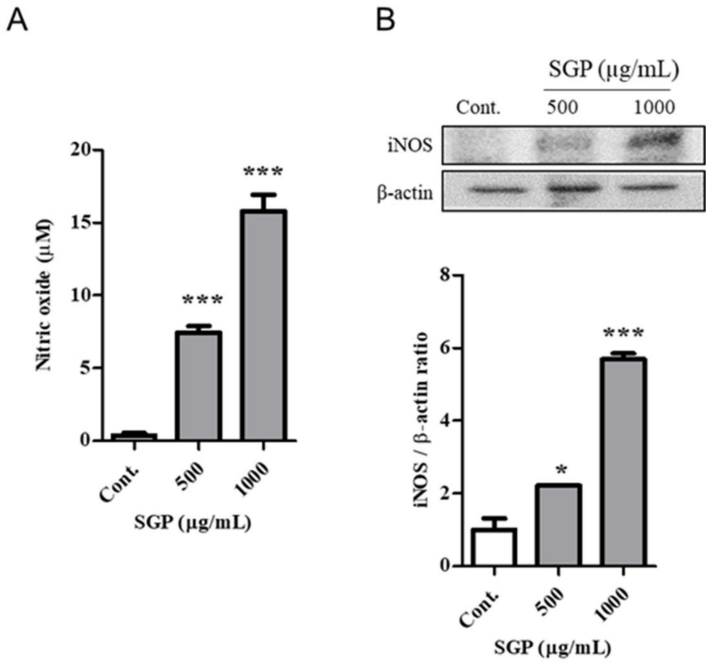

Figure 4. Effects of SGP on NO and iNOS levels in RAW264.7 macrophages. RAW264.7 macrophages were inserted into 500 and 1000 μg/mL SGP for 22 h (for NO) or 18 h (for iNOS). After 22 h, cell supernatants were used for NO production analysis. Supernatants were used according to the Griess method (**A**). After 18 h, cells were collected to evaluate iNOS protein levels; expression levels were determined through immunoblotting and quantification of iNOS/β-actin intensity (**B**). Shown are the means ± SD of triplicate experiments. * $p < 0.05$ and *** $p < 0.0001$, compared to the controls.

3.5. SGP Increased Immune-Enhancing Activity through Phosphorylation of MAPKs and NF-κB Signaling Pathways in RAW264.7 Macrophages

To investigate whether SGP has an effect on the MAPK phosphorylation in RAW264.7 macrophages, the phosphorylation levels of ERK, JNK, and p38 were evaluated by immunoblotting. JNK, ERK, and p38 are members of the MAPK, and when MAPKs are activated, they regulate cell functions including cell growth, gene expression, and differentiation [33]. In addition, they can activate macrophages by phosphorylating transcription factors. Our results confirmed JNK, ERK, and p38 MAPK signaling pathway phosphorylation in macrophages activated by SGP. We also found that SGP activated the NF-κB signaling pathway. NF-κB is an essential expression factor which regulates genes involved in innate and adaptive immune responses [34]. RAW264.7 cells were inserted into SGP at each concentration (500 and 1000 μg/mL) for 30 min. Immunoblotting bands were quantified and expressed as the ratios of p-ERK/ERK, p-JNK/JNK, p-p38/p38, and p-p65/p65 intensity. Phosphorylation of p38, ERK, JNK, and p65 was significantly increased by SGP treatment (500 and 1000 μg/mL) in RAW264.7 macrophages (Figure 6A). The quantification graphs of p-ERK, p-JNK, p-p38, and p-p65/p65 intensity show an SGP for each concentration (500 and 1000 μg/mL) in RAW264.7 macrophages (Figure 6B–E).

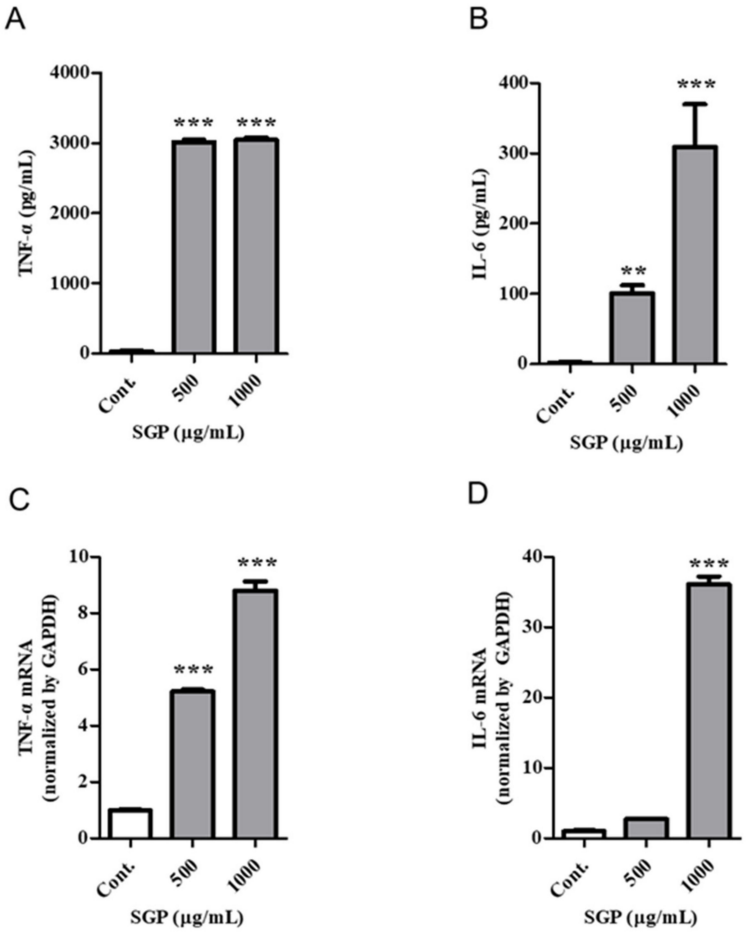

Figure 5. Effect of SGP on cytokine production and mRNA levels of IL-6 and TNF-α in RAW264.7 macrophages. RAW264.7 cells were stimulated with SGP at 500 and 1000 μg/mL for 24 h. Production of IL-6 and TNF-α was determined using an ELISA (**A**,**B**). RAW264.7 cells were stimulated with SGP at 500 and 1000 μg/mL for 12 h, and IL-6 and TNF-α mRNA levels were determined using RT-qPCR (**C**,**D**). Shown are the means ± SD of triplicate experiments. ** $p < 0.001$ and *** $p < 0.0001$, compared to the controls.

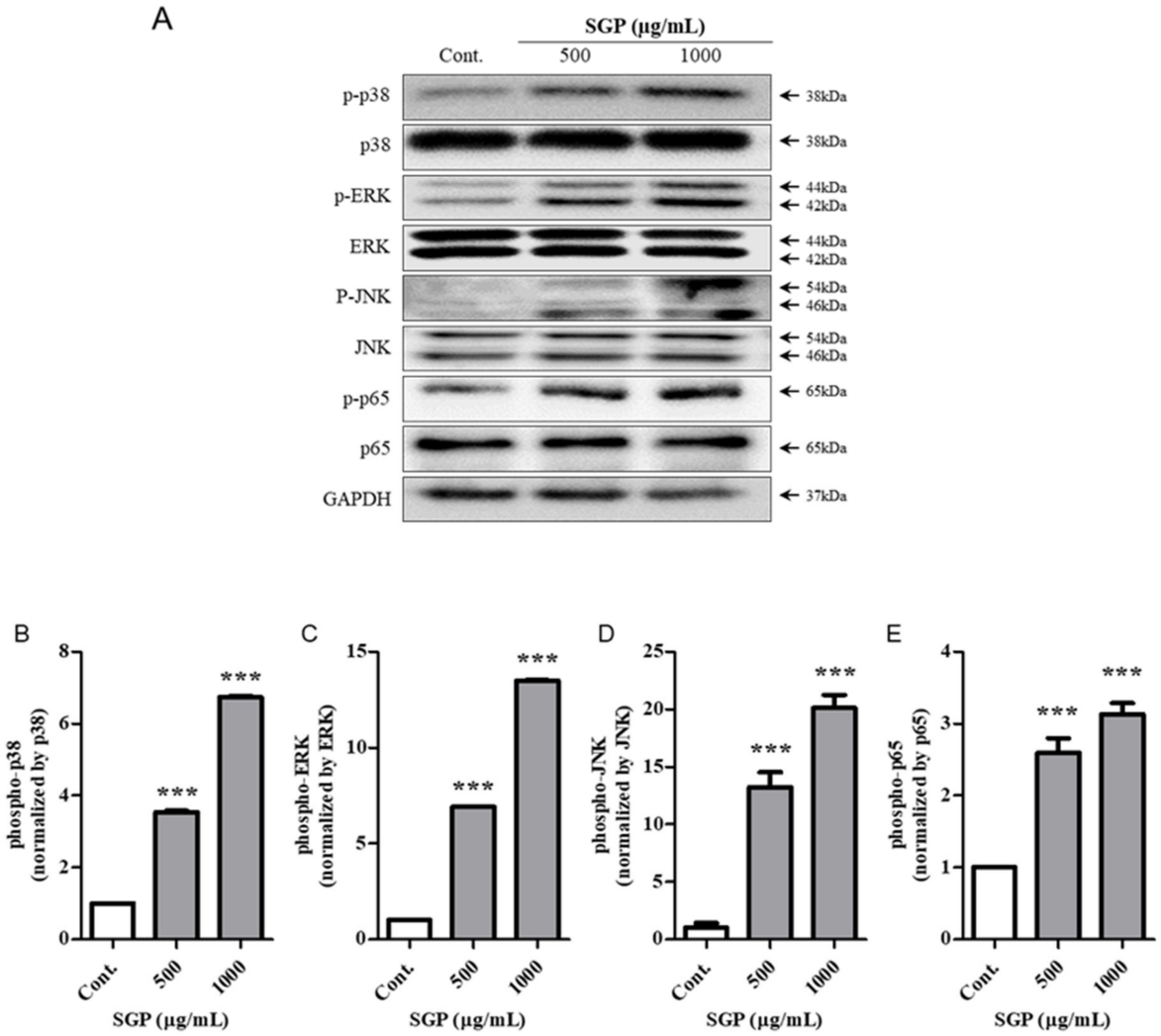

Figure 6. Phosphorylation of MAPKs and NF-κB through SGP stimulation of RAW264.7 cells. RAW264.7 cells were stimulated with SGP (500 and 1000 μg/mL) for 30 min. Phosphorylation and total protein detection of ERK, JNK, p38, and p65 from cell lysates prepared from SGP-stimulated RAW264.7 cells were calculated by phosphor-specific or total protein antibodies. Phosphorylation or total protein levels of ERK, JNK, p38, and p65 (**A**). Quantification of p-ERK/ERK, p-JNK/JNK, p-p38/p38, and p-p65/p65 intensity (**B**–**E**). Shown are the means ± SD of triplicate experiments. *** $p < 0.0001$, compared to the controls.

4. Conclusions

Botanical polysaccharides present various beneficial therapeutic properties, and it is thought that the mechanisms involved in these effects are due to the modulation of innate immunity and, more specifically, macrophage function [35–37]. In this study, we focused on immune-enhancing effects of polysaccharide fraction (SGP) obtained from heat-processed ginseng using RAW264.7 macrophages. The production of IL-6, TNF-α, and NO and phosphorylation of MAPK and NF-κB in SGP-activated macrophages may contribute to enhanced innate immune functions (Figure 7). However, more studies on the linkage analysis and structure-activity relationship of the active polysaccharide must elucidate further study. These findings indicate the potential usefulness of SGP as an immunostimulant and a promising therapeutic candidate for further development.

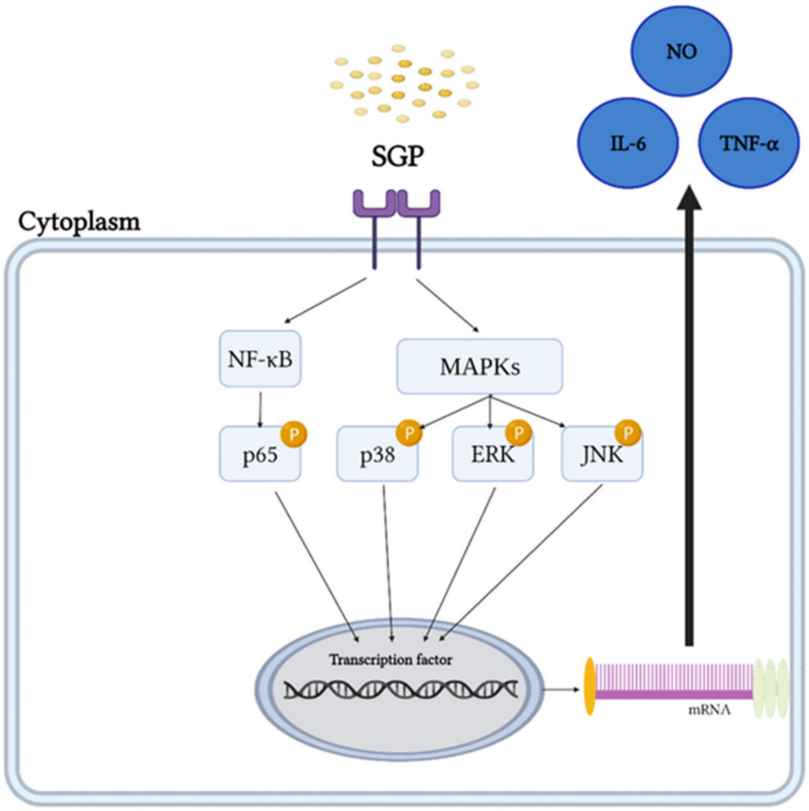

Figure 7. Schematic diagram of MAPKs and NF-κB signaling pathways on SGP treatment in RAW264.7 macrophages.

Author Contributions: Conceptualization, M.-S.S., K.S.K. and S.-H.B.; methodology, S.J.K. and M.K.; validation, S.J.K. and M.K.; investigation, S.J.K., M.-S.S. and M.K.; resources, M.K. and S.-H.B.; writing—original draft preparation, S.J.K. and M.K.; writing—review and editing, M.-S.S., K.S.K. and S.-H.B.; funding acquisition, S.-H.B. and K.S.K. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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