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Immunostimulatory activity of polysaccharides from *Cheonggukjang*

Seung-Jun Lee [a,b,1], Hong-Kun Rim [a,1], Ji-Yun Jung [a,b], Hyo-Jin An [a,2], Ji-Sun Shin [a], Chang-Won Cho [c], Young Kyoung Rhee [c], Hee-Do Hong [c], Kyung-Tae Lee [a,b,*]

[a] *Department of Pharmaceutical Biochemistry, College of Pharmacy, Kyung Hee University, Dongdaemun-Ku, Hoegi-Dong, Seoul 130-701, Republic of Korea*
[b] *Department of Life and Nanopharmaceutical Science, Kyung Hee University, Dongdaemun-Ku, Hoegi-Dong, Seoul 130-701, Republic of Korea*
[c] *Korea Food Research Institute, Kyunggi, Seongnam 463-836, Republic of Korea*

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ABSTRACT

Cheonggukjang is a Korean whole soybean paste fermented by *Bacillus subtilis* and regarded as a healthy food. The objective of this study was to investigate the immunostimulatory activity of polysaccharides from *Cheonggukjang* (PSCJ) in RAW 264.7 macrophages and an animal model. PSCJ induced mRNA expressions of inducible nitric oxide synthase and tumor necrosis factor-α (TNF-α) by activating nuclear factor-κB, and subsequently increased the productions of nitric oxide (NO) and TNF-α in murine recombinant interferon-γ-primed RAW 264.7 macrophages. Furthermore, after daily oral administration of PSCJ, immobility time decreased significantly in the PSCJ-administered group (200 or 400 mg/kg) on day 10. Taken together, these results suggest that the PSCJ has a possible role improving immune function through regulatory effects on immunological parameters, such as NO and TNF-α productions and changes in indicators related to fatigue.

1. Introduction

Macrophages and lymphocytes play a major role in host defense as part of the non-specific defense (innate immunity) and specific defense (adaptive immunity) systems. Macrophages are the first cells to recognize infectious agents and are central to cell-mediated and humoral immunity. Activated macrophages increase production of nitric oxide (NO) and cytokines, which function as mediators of the immune system. NO is an important intra- and inter-cellular regulatory molecule with multiple biological functions, including macrophage-mediated cytotoxicity (Moncada et al., 1991). Tumor necrosis factor-α (TNF-α) is also a significant regulator of the inflammatory and immune responses. These molecules are involved in the nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways. The majority of the spleen's functions are related to the immune system. The spleen stores and produces lymphocytes that produce antibodies and assist in removing microbes and other debris from the blood (Brown et al., 2003). Lymphocytes are a type of white blood cell in the vertebrate immune system and can be divided into natural killer (NK) cells, T cells, and B cells. NK cells are part of the innate immune system and play a major role defending the host from both tumors and virally infected cells. T and B cells are the major cellular components of the adaptive immune response (Vivier et al., 2011).

The forced swimming test (FST) is a behavioral despair test used to measure the effect of antidepressant drugs on the behavior of laboratory animals (Porsolt et al., 1977) and is also used as an anti-fatigue and endurance test. This test induces immobility as a reflection of helplessness when animals are subjected to an inescapable situation (tank of deep water). In this paradigm, mice are placed in a tank for an extended period. After an initial swimming period, the animal exhibits immobility behavior considered a fatigue-like response. Therefore, the FST is used to examine whether a certain agent has anti-fatigue and immune-enhancing effects (Kumar and Garg, 2009). Blood urea nitrogen (BUN), creatine kinase (CK) and lactic dehydrogenase (LDH) are blood biochemical parameters related to fatigue (Shin et al., 2004). Glucose levels decrease

Abbreviations: BUN, blood urea nitrogen; CK, creatine kinase; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; ELISA, enzyme linked immunosorbent assay; ERK, extracellular-signal-related kinase; FBS, fetal bovine serum; FST, forced swimming test; IFN-γ, interferon-γ; IκB-α, inhibitory κB-α; IL-2, interleukin-2; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; LDH, lactic dehydrogenase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; NO, nitric oxide; PARP-1, poly [ADP-ribose] polymerase-1; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonylfluride; PSCJ, polysaccharides from *Cheonggukjang*; RPMI, rosewell park memorial institute medium; SDS, sodium dodecyl sulfate; TNF-α, tumor necrosis factor-α; TP, total protein.

* Corresponding author at: Department of Pharmaceutical Biochemistry, College of Pharmacy, Kyung Hee University, Dongdaemun-Ku, Hoegi-Dong, Seoul 130-701, Republic of Korea. Tel.: +82 2 961 0860; fax: +82 2 966 3885.
E-mail address: ktlee@khu.ac.kr (K.-T. Lee).

[1] These authors contributed equally to this work.

[2] Present address: Department of Pharmacology, College of Oriental Medicine, Sangji University, Wonju-si, Gangwon-do 220-702, Republic of Korea.

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immediately after exercise (Wagenmakers et al., 1991), and total protein (TP) measurements can reflect nutritional condition, liver disease, kidney disease, and many other conditions (Dorchy, 2002).

Various fermented soybean products, such as Natto, Doenjang, and Tempeh are prepared in Asia. *Cheonggukjang* is a soybean paste fermented by *Bacillus subtilis*. It is regarded as a good source of protein, hydrolyzed peptides, and lipids and is consumed commonly by Koreans for its health benefits, such as a reduction in arterial stiffness. *Cheonggukjang* is also characterized by its unique flavor and sticky, fibrous texture, due to γ-aminobutyric acid formed during fermentation (Cho et al., 2009). *Cheonggukjang* has antioxidative (Kim et al., 2011), free-radical-scavenging, tyrosinase-inhibitory (Choi et al., 2008), and anti-microbial activities (Kim et al., 2004). In addition, *Cheonggukjang* exhibits fibrinolytic activity (Ko et al., 2008) and anticancer properties (Seo et al., 2009). Soybean mucilage, traditional Deonjang and *Bacillus* spp. strains isolated from *Cheonggukjang*, has immunostimulatory activity (Chang et al., 2005; Lee et al., 2011; Xu et al., 2006). However, few studies have reported on its immunostimulatory activity or the molecular mechanisms involved. Therefore, we investigated the immunostimulatory activity and underlying molecular mechanisms of the polysaccharides from *Cheonggukjang* (PSCJ) on recombinant interferon (rIFN)-γ-primed RAW 264.7 macrophages and primary splenocytes, and, subsequently in a FST animal model.

2. Materials and methods

2.1. Materials and chemicals

Soy beans were purchased from Namboeun NongHyup (Boeun, Korea) and commercial *Cheonggukjang* starter culture (*B. subtilis* 10%, soybeans 90%) was purchased from NUC corp. (Daegu, Korea). Dulbecco's modified Eagle medium (DMEM), rosewell park memorial institute medium (RPMI), fetal bovine serum (FBS), penicillin and streptomycin were obtained from Life Technologies (Grand Island, NY, USA). Inhibitory κB-α (IκB-α), iNOS, p65, poly [ADP-ribose] polymerase-1(PARP-1), extracellular-signal-related kinase (ERK) and β-actin monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). p-IκB-α, p-p65, c-Jun N-terminal kinase (JNK) and p38 monoclonal antibodies were purchased from Cell Signaling Technology (Boston, MA, USA). CD3 and CD28 antibodies were purchased from BD pharmingen (San Diego, CA, USA). Enzyme linked immunosorbent assay (ELISA) kits for TNF-α, interleukin-2 (IL-2), and interferon-gamma (IFN-γ) were obtained from R&D Systems (Minneapolis, MN, USA). Dimethyl sulfoxide (DMSO), sulfanilamide, phenylmethylsulfonylfluride (PMSF), dithiothreitol (DTT), murine recombinant IFN-γ, lipopolysaccharide (LPS), sodium bicarbonate, HEPES, sodium dodecyl sulfate (SDS), polymyxin B sulfate and all other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Preparation of PSCJ

Whole soybeans (1000 g) were washed 5–6 times and soaked with tap water at 4 °C for 24 h, and steamed for 40 min at 121 °C. The steamed soybeans were left to stand at 50 °C to cool down. Then, the cooked soybeans were inoculated with 0.5% (w/w) *Cheonggukjang* starter culture and fermented for 48 h at 40 °C in an incubator. The *Cheonggukjang* samples were freeze-dried and stored at −20 °C. Lyophilized *Cheonggukjang* powder were extracted with 10 volumes of distilled water at 90 °C for 3 h. Extracts were centrifuged at 6500*g* for 20 min and filtered through Advantec filter paper No. 2 (5 μm, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) to remove insoluble matter. Polysaccharides were precipitated from resuspended extracts using 95% ethanol by adjusting to final concentration of 80% ethanol. The precipitate was dissolved in distilled water and dialyzed (M.W. > 6000) against distilled water for 3 days. After removal of insoluble materials in the dialysis, the supernatant was lyophilized. The lyophilised powder (denoted as PSCJ) was stored in dark at 4 °C.

2.3. Characterization of PSCJ

2.3.1. Determination of the contents of total sugar and protein

The neutral sugar and acidic sugar contents of PSCJ were colorimetrically measured using the phenol–sulfuric acid method (Dubois et al., 1956) and the *m*-hydroxybiphenyl method (Blumenkarntz and Asboe-Hansen, 1973), respectively. Galactose and galacturonic acid were used as standard materials, respectively. The protein content was determined by partially modified the Bradford method (Bradford, 1976), and bovine serum albumin was used as standard material.

2.3.2. Analysis of neutral sugar composition in PSCJ

The analysis of neutral sugar composition was performed as follows by partially modified method of Albersheim (Thomas and Albersheim, 1972). Briefly, 2 M trifluoroacetic acid was added to the polysaccharide samples and hydrolyzed at 121 °C for 90 min, and then dissolved in 1 M ammonia solution and reduced with 10 mg $NaBH_4$ for 4 h; a moderate amount of acetic acid was added to eliminate the remaining $NaBH_4$; methanol was then added for repeated drying to eliminate the excess acetic acid and to convert into alditols for the corresponding component sugars. Each alditol was reacted with 1 ml acetic anhydride at 121 °C for 30 min to be converted into alditol acetate, which was then separated and extracted using the 2-phase solvent system of chloroform/H_2O. The extract was dried and subsequently dissolved in a small amount of acetone for analysis by GC (ACME-6100, Young-Lin Co., Ltd., Anyang, Korea). The GC column was an SP-2380 capillary column (0.25 mm × 30 cm, 0.2 μm film thickness, Supelco, Bellefonte, PA, USA), and the detector was a flame ionization detector (FID, Young-Lin Co., Ltd.). The carrier gas was N_2 with flow rate of 1.5 ml/min. The injection temperature was 250 °C, and the detector temperature was 270 °C; the column temperature was increased by 30 °C/min from 60 °C to 220 °C and by 8 °C/min from 220 °C to 250 °C. The mole% of each component sugar was calculated from the peak area of each derivative, molecular weight, and molecular response factor for FID.

2.4. Assay of immunostimulatory activity of PSCJ

2.4.1. Cell culture and sample treatment

RAW 264.7 macrophages were obtained from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in DMEM containing 10% FBS, penicillin, and streptomycin sulfate at 37 °C in a 5% CO_2 atmosphere. Cells were pretreated with rIFN-γ (100 ng/ml) and then stimulated with various concentrations of PSCJ (12.5, 25, or 50 μg/ml) or LPS (1 μg/ml) for the indicated time.

2.4.2. Splenocytes isolation and preparation

Spleens were excised aseptically from sacrificed animals and placed in RPMI. Single cell suspensions were prepared, passed through a 70-μm-pore mesh, and washed with RPMI. Samples were spun (800 rpm, room temperature, 5 min) and supernatants were aspirated. In order to lyse the erythrocytes, the pellets were resuspended in Red Blood Cell Lysing Buffer Hybri-Max™ (Sigma), incubated for 3 min, and then washed twice with RPMI. Pellets were resuspended in RPMI (containing 10% FBS) and the number of cells/ml was measured.

2.4.3. MTT assay

RAW 264.7 macrophages were seeded in 96-well plates and stimulated with the PSCJ. Cells were incubated with a MTT solution for 4 h at 37 °C under 5% CO_2. The MTT-containing medium was removed and the cells were solubilized in DMSO. Absorbance of each well at 540 nm was measured using a microplate reader (Molecular Devices Inc., Sunnyvale, CA, USA).

2.4.4. MTS/PMS assay

Splenocytes were incubated with a MTS/PMS dye mixture (Promega, Madison, WI, USA) was added and incubated for 4 h at 37 °C under 5% CO_2. After incubation, the optical density (OD) of formazan product was measured at 490 nm using a microplate reader.

2.4.5. Nitrite assay

RAW 264.7 macrophages (1×10^5 cells/ml) were cultured in 24-well plates and stimulated with the PSCJ. After 24 h, culture supernatants were collected and nitrite was measured using Griess reagent. Equal volumes of Griess reagent (1:1 of 0.1% N-1 naphthylethylenediamine in 5% phosphoric acid and 1% sulfanilamide in 5% phosphoric acid) and sample were incubated together at room temperature for 10 min. Absorbance at 540 nm was measured using a microplate reader.

2.4.6. Determination of TNF-α, IL-2, and IFN-γ productions

TNF-α, IL-2, and IFN-γ levels in cell culture media were quantified using ELISA kits, according to the manufacturer's instructions.

2.4.7. Western blot analysis

The cells were collected by centrifugation and washed once with phosphate buffered saline (PBS). Washed cell pellets were resuspended in protein extraction solution PRO-PREP (Intron Biotechnology, Seoul, Korea) and then incubated for 15 min at 4 °C. Cell debris was removed by microcentrifugation and supernatants were quick frozen. The protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories Inc, Hercules, CA, USA) according to the manufacturer's instruction. Proteins (40 μg) were electroblotted onto a PVDF membrane following separation on a 10% SDS-polyacrylamide gel electrophoresis. The immunoblot was incubated for 1 h with blocking solution (5% skim milk) at room temperature, and then incubated overnight with a 1:1000 dilution of primary antibody at 4 °C. Blots were washed three times with Tween 20/Tris-buffered saline (T/TBS) and then incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA)