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Cheonggukjang polysaccharides enhance immune activities and prevent cyclophosphamide-induced immunosuppression

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ABSTRACT

Cheonggukjang is a traditional Korean fermentation product prepared from soybean that is reported to have various biological functions. We previously reported that the polysaccharides from *Cheonggukjang* (PSCJ) have immunostimulatory activities in RAW 264.7 macrophages and primary cultured splenocytes. In this study, the immunostimulatory activities of the PSCJ were investigated further using various experimental models such as *in vitro*, *ex vivo*, and *in vivo*. The PSCJ was able to stimulate the complement system ($ITCH_{50}$: 30.6%). In primary cultured mouse peritoneal macrophages, the PSCJ was found to significantly increase nitric oxide and immunostimulatory cytokines (IL-6 and IL-12) production in a concentration-dependent manner (1–100 μg/mL). In the normal mice model, the oral administration of the PSCJ increased the weight of spleen ($p < 0.05$ at 100 and 200 mg/kg) and improved the phagocytic rates of peritoneal macrophages ($p < 0.05$ at 200 mg/kg) and lymphocytes proliferation ($p < 0.05$ at 100 and 200 mg/kg). Similarly, the PSCJ markedly restored the decreased lymphocytes proliferation ($p < 0.01$ at 200 mg/kg), natural killer cell activity ($p < 0.01$ at 200 mg/kg), and white blood cell count ($p < 0.01$ at 100 and 200 mg/kg) in the cyclophosphamide-induced immunosuppressed mice model. These results suggest that the PSCJ could be utilized as an effective immunostimulatory agent.

1. Introduction

The modulation of immune response to prevent diseases has long been one of the primary concerns of many researchers [1]. It was recently proven that the use of immunomodulators to enhance the host defense responses can be an effective way to increase resistance to disease [2]. Polysaccharides separated from natural substances including food ingredients and medicinal plants have been regarded as important immunostimulant candidates due to their broad spectrum of immunostimulatory activities with relatively low toxicity and adverse effects [3]. Polysaccharides from natural sources have been known to improve the immune functions of the body by activating immune-related cells, such as macrophages, lymphocytes, and natural killer (NK) cells, and by promoting cytokine secretion, antibody production, and activation of the complement system [4]. Therefore, many natural polysaccharides are receiving increasing attention in the fields of therapeutics and functional foods.

Cheonggukjang is a traditional Korean fermentation product prepared from soybean and is also called Natto, Tempeh, and Douchi in other Asian countries [5]. *Cheonggukjang* is manufactured by the short-term fermentation of soybeans using *Bacillus subtilis*, which contains many enzymes, microorganisms, and bioactive compounds that are absent from unfermented soybeans [6]. *Cheonggukjang* is known for its functionality, including antioxidant, anti-inflammatory, anti-hypertensive, and anti-diabetic activities [7], in addition to its nutritional value. Most previous functionality studies of *Cheonggukjang* used the whole product or a crude extract [5–8]. However, few studies have reported the activity of polysaccharides from *Cheonggukjang*. In the studies that investigated the biological activities of polysaccharides prepared from the traditional Korean fermentation products, it was reported

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that the polysaccharides from persimmon vinegar, soy sauce, and *makgeolli*, which is traditional Korean rice wine, have an effect on immune activity [9–11].

In a previous study, we isolated and characterized polysaccharides from *Cheonggukjang* (PSCJ), and investigated their immunostimulatory activity and underlying molecular mechanisms on recombinant interferon (rIFN)-γ-primed RAW 264.7 macrophages and primary cultured splenocytes [12]. To further investigate the immunostimulatory activities of the PSCJ, the present study explores the effect of the PSCJ on the complement system and primary cultured peritoneal macrophages and subsequently in normal mice and cyclophosphamide (CY)-induced immunosuppressed mice models.

2. Materials and methods

2.1. Materials and chemicals

Soybeans were purchased from Namboeun NongHyup (Boeun, Korea) and commercial *Cheonggukjang* starter culture (*B. subtilis* 10% and soybeans 90%) was purchased from NUC Corp. (Daegu, Korea).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), concanavalin A (ConA), dimethyl sulfoxide (DMSO), Griess reagent, lipopolysaccharide (LPS), phosphate buffered saline (PBS), thioglycollate, and trypan blue solution (0.4%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). CVT-E002™ (sold commercially as COLD-FX®) was purchased from Afexa Life Sciences Inc. (Edmonton, AB, Canada). Rosewell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from GIBCO BRL (Grand Island, NY, USA). Enzyme linked immunosorbent assay (ELISA) kits for interleukin (IL)-6 and IL-12 were obtained from BD Biosciences (San Diego, CA, USA). Cyclophosphamide (CY) was purchased from Jiangsu Heungrui Medicine Co., Ltd. (Lianyungang, China).

2.2. Preparation of the PSCJ

Whole soybeans (1000 g) were washed five to six times and soaked with filtered water at 4 °C for 24 h and then steamed for 40 min at 121 °C. The steamed soybeans were maintained at 50 °C to allow them to cool down. The cooked soybeans were then 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 was extracted with 10 volumes of distilled water at 90 °C for 3 h. The 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 any insoluble particles. Polysaccharides were precipitated from the extracts by the addition of four volumes of 95% ethanol. The precipitate was dissolved in distilled water and dialyzed using Spectra/Por (MWCO; 6000–8000, Spectrum Laboratories Inc., Rancho Dominquez, CA, USA) for three days. Finally, the high-molecular-weight solution was lyophilized. The lyophilized powder (denoted as PSCJ) was stored in the dark at 4 °C.

2.3. Anti-complementary activity of the PSCJ (in vitro)

The anti-complementary activity was measured by the complement fixation test based on complement consumption and the degree of red blood cell lysis by residual complement [13]. Normal human serum (NHS) was obtained from volunteer adults. A 50 μL aliquot of 1000 μg/mL PSCJ was mixed with equal volumes of NHS and gelatin veronal-buffered saline (GVB^{2+}, pH 7.4) containing 500 mM Mg^{2+} and 150 mM Ca^{2+}, respectively. The mixtures were pre-incubated at 37 °C for 30 min and the residual total hemolytic complement (TCH_{50}) was determined using IgM hemolysin-sensitized sheep erythrocytes (1×10^8 cells/mL). The NHS was incubated with water and GVB^{2+} to provide a control. The anti-complementary activity of the PSCJ is expressed as the percent inhibition of the control TCH_{50} ($ITCH_{50}$) of polysaccharide K (PSK), which is a known immunoactive polysaccharide from *Coriolus versicolor* that was used as a positive control [14]. $ITCH_{50}$ (%) = [TCH_{50} (control) − TCH_{50} (treated with sample)]/TCH_{50} (control) × 100.

2.4. Immunostimulatory activity of the PSCJ on primary cultured mouse peritoneal macrophages (ex vivo)

2.4.1. Primary culture and cytotoxicity test

Specific pathogen-free (SPF), 5-week-old male C57BL/6 mice were purchased from Hanlim Experimental Research Institute (Hwaseong, Korea). The peritoneal macrophages of C57BL/6 mice were harvested from 5% thioglycollate-treated mice as described previously [15]. The peritoneal macrophages were resuspended in RPMI 1640–FBS (containing 10% FBS) medium and then plated into 96 well-culture plates (2×10^5 cells/well). After incubation for 2 h, the non-adherent cells were removed by PBS washing, and the adherent macrophages were stimulated with various concentrations of the PSCJ for 24 h. The macrophages 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. The absorbance of each well at 540 nm was measured using a microplate reader (TECAN, Männedorf, Switzerland).

2.4.2. Quantification of nitric oxide and cytokine production

Peritoneal macrophages were seeded in 96-well plates (2×10^5 cells/well) and stimulated with various concentrations of the PSCJ. After 24 h, the culture supernatants were collected, and the nitric oxide (NO) concentration was measured using the Griess reagent. Equal volumes of the Griess reagent (1% sulfanilamide, 0.1% naphthylenediamine dihydrochloride, and 0.5% H_3PO_4) and the sample were incubated together at room temperature for 10 min. The absorbance at 540 nm was measured using a microplate reader (TECAN). The concentrations of cytokines (IL-6 and IL-12) in the culture supernatants were determined using ELISA kits according to the manufacturer's protocol.

2.5. Immunostimulatory activity of the PSCJ in vivo

2.5.1. Animals

SPF KM male mice (7–8 weeks of age) were obtained from the Experimental Animal Center of Yanbian University College of Medicine (Yanji, China). The mice were maintained under constant conditions (temperature: 22 ± 2 °C, humidity: 40–60%, light and dark cycle: 12 h) and allowed free access to water and food. All of the animal experiments were performed according to the instructions of the Medical Ethics Committee for the Use of Experimental Animals at Yanbian University.

2.5.2. Animal experiments

The animal experiments were conducted using both normal and immunosuppressed mice.

For the animal experiments with normal mice, the mice were randomly divided into three groups (ten mice in each group). From day 1 to 28, the three different groups of mice were orally treated with saline or the PSCJ at 100 and 200 mg/kg body weight on a daily basis. Twenty-four hours after the last administration, the mice were weighed and sacrificed by cervical dislocation. The spleen and thymus were immediately removed and weighted. The spleen and thymus index were calculated as the spleen and thymus weight/body weight, respectively. The spleen sample was used for lymphocyte proliferation.