Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Immunomodulatory effects of polysaccharide fraction isolated from *Fagopyrum esculentum* on innate immune system

Mi-Jin Oh [a], Hee-Don Choi [b], Sang Keun Ha [a], Inwook Choi [a, **], Ho-Young Park [a, *]

[a] Division of Functional Food Research, Korea Food Research Institute, Jeollabuk-do 55365, Republic of Korea
[b] Division of Strategic Food Research, Korea Food Research Institute, Jeollabuk-do 55365, South Korea

ARTICLE INFO

Article history:
Received 17 January 2018
Accepted 29 January 2018
Available online xxx

Keywords:
Fagopyrum esculentum
Polysaccharides
Immunomodulatory activity
Macrophage
Natural killer cell
Cyclophosphamide

ABSTRACT

The present study investigates the immunomodulatory activities of buckwheat polysaccharide fraction (BPF) from the seed of *Fagopyrum esculentum* on RAW 264.7 macrophage cell line and Cyclophosphamide-induced immunosuppressed conditions in mice models. The results of *in vitro* showed that treatment with 0.5−10 μg/mL of BPF can modulate immune responses. MTT assay and nitric oxide production and immune-related cytokine levels were conducted. Treatment with BPF at a dose of 10 μg/mL of BPF increased immune responses on macrophages. Moreover, natural killer (NK) cell cytotoxicity was conducted. The apoptosis of YAC-1 cells increased as the co-culture ratio between spleen cells and YAC-1 cells increased approximately 4- fold compared to the control group from 12.5:1 to 50.0:1. The *in-vivo* immunomodulatory effects of BPF were evaluated by cyclophosphamide-induced mice model. The immune response of BPF was determined against cyclophosphamide (100 mg/kg) immunosuppressed mice at doses of 50 mg/kg and 100 mg/kg of BPF as compared to control. The results of this study showed that BPF administration increased spleen and thymus indices as well as the leukocytes count in the blood of immunosuppressed mice. All of results suggested that BPF are potentially acts as immunomodulator for activation of immune responses.

1. Introduction

The immune system is a tightly defensive mechanism of human to protecting from pathogens, injury, infections and contaminants. Immune responses are mediated by innate and adaptive immune systems to engagement with invading agents [1].

Immunomodulation is a complex mechanism involved in immunosuppression and immunostimulation. Immunosuppression is a reduction of the immune responses and related the drugs or agents can be modulated the side effects [2]. Immunosuppressors also have been the modulation of various inflammatory diseases or autoimmune disorders [3].

Cyclophosphamide (CTX) is generally used on the World Health Organization's list of chemotherapeutic agents for a human health with safety and cost-effectiveness [4]. CTX is used for various cancer and immune disorders. It was chemotherapy drugs and significant effects of stimulating immune responses [5]. Researchers had studied immunomodulators to reduced toxicity and enhanced defense responses against infectious agents [6].

Buckwheat is one of the most versatile crops with a traditional cultivation range stretching from Eastern Europe to Japan. Because of its strong ability to adapt to various environmental conditions, two main species of buckwheat are cultivated worldwide. Common buckwheat (*Fagopyrum esculentum*) is grown widely countries such as Japan, Nepal, China, Canada and USA. Tartary buckwheat (*Fagopyrum tataricum*) has been only found in the mountainous regions of China [7]. Buckwheat was functional foods and advantageous for most human health. Various tissues of buckwheat contain multiple nutritional compounds such as high level of proteins in its grains and a variety of vitamins and amino acids in its seeds [8].

Buckwheat polysaccharide is an important functional ingredient of buckwheat [9]. There are few studies on buckwheat polysaccharides improved the treatment of diseases. Further studies have attempted to clarify the mechanism of disorders using the buckwheat polysaccharides. Carbohydrates include many molecules such as monosaccharides, disaccharides, oligosaccharides and

* Corresponding author. Korea Food Research Institute, 245, Nongsaengmyeong-ro, Iseo-myen, Wanju-gun, Jeollabuk-do 55365, Republic of Korea.
** Corresponding author. Korea Food Research Institute, 245, Nongsaengmyeong-ro, Iseo-myen, Wanju-gun, Jeollabuk-do 55365, Republic of Korea.
E-mail addresses: choiw@kfri.re.kr (I. Choi), hypark@kfri.re.kr (H.-Y. Park).

https://doi.org/10.1016/j.bbrc.2018.01.172
0006-291X/

polysaccharides. Among them polysaccharide is polymers composed of many sugar building blocks. Plant polysaccharide provides many benefits to the body to relatively low toxicity and side effects along with increases immunity and overall health. Thus, polysaccharides are a great source of immunomodulatory function [10].

In this study, we evaluated the immunomodulatory activities of polysaccharide fraction isolated from buckwheat (BPF) on the enhancement of immune response. Immunomodulatory activities of murine macrophages and natural killer (NK) cells related to immune reactions, such as cytotoxicity and nitric oxide (NO) production were assessed. The *in vivo* immunomodulatory activity of BPF was evaluated using CTX-induced immunosuppression models by measuring immune organ index and hematological parameters.

2. Materials and methods

2.1. Preparation of BPF

Buckwheat (*F. esculentum*) collected from Hongcheon-gun, Republic of Korea. Buckwheat (100 g) seed was extracted with 2 L of distilled water at 80 °C for 3 h, at a ratio of 1:20 (w/v). The aqueous extract was filtered and concentrated to 200 mL at 40 °C using a vacuum rotary evaporator. It was precipitated by adding four volumes of 95% ethanol and added to concentrate large molecules, such as polysaccharides, overnight at 4 °C. The precipitates were pelleted by centrifugation at 4000 × g for 30 min and then re-dissolved in 70% ethanol with centrifugation at 4000 × g for 30 min. The precipitates were re-dissolved in distilled water and lyophilized to obtain BPF. The yield of dried extract from the crude material was 6.6%.

2.2. Characterization of BPF

2.2.1. Total sugar and protein analysis

Total sugar content of BPF was determined using the phenol-sulfuric acid colorimetric method with D-glucose as the standard. Briefly, 0.5 mL of BPF was mixed with the same volume of 5% phenol, and by adding five volumes of concentrated sulfuric acid was added. The mixture was reacted for 20 min, and the absorbance was measured at 470 nm using a microplate reader (Spectra MAX M2, Molecular Device, Sunnyvale, CA, USA). The protein content of BPF was determined using the Bradford method with bovine serum albumin as a standard, and the absorbance was measured at 595 nm.

2.2.2. Total polyphenol analysis

Total polyphenol content of BPF was determined using the Folin-Denis method [11] with garlic acid as the standard. Briefly, 0.5 mL of BPF was mixed with the same volume of 10% 2 N Folin−Ciocalteu' phenol reagent react for 3 min at room temperature, after 0.5 mL of 20% Na_2CO_3 solution was added. The mixtures were kept in the dark place at room temperature for 1 h, and the absorbance was measured at 750 nm. The results are expressed as mg garlic acid equivalents (GAE)/g dry weight.

2.2.3. KDO-like material analysis

2-keto-3-deoxy-D-manno-2-octulosonic acid (KDO) content of BPF was detected via a modification of the methods [12]. BPF and 0.4 N sulfuric acid were mixed and incubated at around 100 °C for 30 min and cooled in an ice bath. Sodium arsenite (2%, w/v in 0.5 N HCl) was added and waited to stand until the yellow color for 2−3 min. Thiobarbituric acid (0.6%, w/v) was added and shaken the solution was heated to 100 °C for 15 min. The pink solution was cooled to 4 °C and the absorbance was measured at 548 nm.

2.2.4. High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

Dionex Bio-LC DX-300 system (Dionex, Sunnyvale, CA, USA) was used the separated of sugars in BPF. The column was CarboPac-PA100 guard column and CarboPac-PA100 analytical column was used HPAEC-PAD. Eluents were prepared by 18 mM NaOH/200 mM NaOH and elution was carried out at a flow rate of 0.1 mL/min and injection volume was 25 μl. The standard solutions used fucose, rhamnose, arabinose, galactose, glucose, and xylose were calculated standard curve. Peak areas were interpreted using AI-450 chromatography software (version 3.3.1., Dionex).

2.2.5. Determination of endotoxin contamination

The concentration of endotoxins of BPF was detected using a Pierce Limulus amebocyte lysate (LAL) Chromogenic Endotoxin Quantitation Kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instruction. Briefly, BPF was incubated with LAL reagent at 37 °C for 5 min in sterile 96-well plates, followed by incubation with chromogenic substrate supplied in the kit. The reaction was stopped using 25% acetic acid (Stop Reagent) and absorbance was measured at 405 nm. The concentration of endotoxins in BPF was determined using a standard curve prepared using endotoxin standard solutions.

2.3. In vitro experiments

2.3.1. Cell culture

Murine macrophage RAW 264.7 cells were obtained from Korean Cell Line Bank (Seoul, Republic of Korea). Cells were suspended in DMEM (Gibco, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin, and 100 IU/mL streptomycin in an atmosphere of 5% CO_2 in air at 37 °C.

2.3.2. Determination of viability of RAW 264.7 cells

Cell viability was according to the mitochondria-dependent reduction of MTT method [13]. RAW 264.7 cells were seeded into a density of 5×10^4 cells/well in 96-well flat bottom plates with medium. After pre-culture for 18 h, untreated normal control and various concentrations (0.5 μg/mL, 1 μg/mL, 5 μg/mL, and 10 μg/mL) of BPF were added into each well and stimulated with or without 1 μg/mL of lipopolysaccharides (LPS) in the medium at 37 °C for 24 h. After treatment, the medium was discarded and cells were incubated with MTT solution (500 μg/mL) for 4 h at 37 °C, and then dissolved in 200 μL of dimethyl sulfoxide with shaking for 5 min. The absorbance of each well was read at 570 nm using a microplate reader.

2.3.3. Determination of NO and cytokines production in RAW 264.7 cells

Nitrite (NO_2^-) was measured breakdown product of NO and the determination of nitrite reaction with the Griess reagent. Thus, the nitrite accumulated in culture medium is indicator of NO production [14]. Cells were seeded into a density of 5×10^4 cells/well in 96-well plates and pre-cultured for 18 h, untreated normal control and various concentrations (0.5 μg/mL, 1 μg/mL, 5 μg/mL, and 10 μg/mL) of BPF were added into each well and stimulated with or without 1 μg/mL of LPS in medium at 37 °C for 24 h. The culture supernatant was mixed with same volume of Griess reagent and incubated at room temperature for 15 min. The concentration of nitrite was measured at absorbance at 550 nm and calculated using the $NaNO_2$ dilution standard curve. Furthermore, using cell supernatants, the generation of interleukin (IL)-6 and tumor necrosis factor (TNF)-α in RAW 264.7 cells determined by the enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Minneapolis, MA, USA). Briefly, microplates were coated with antibodies

Please cite this article in press as: M.-J. Oh, et al., Immunomodulatory effects of polysaccharide fraction isolated from *Fagopyrum esculentum* on innate immune system, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.01.172