International Immunopharmacology 17 (2013) 191–197

Contents lists available at ScienceDirect

International Immunopharmacology

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

Immunostimulatory effects and characterization of a glycoprotein fraction from rice bran

Ho-Young Park [a,c], A-Reum Yu [a], In-Wook Choi [a], Hee-Do Hong [b], Kwang-Won Lee [c], Hee-Don Choi [a,*]

[a] Division of Metabolism and Functionality Research, Korea Food Research Institute, Gyeonggi 463-746, Republic of Korea
[b] Division of Convergence Technology, Korea Food Research Institute, Gyeonggi 463-746, Republic of Korea
[c] Division of Food Bioscience and Technology, College of Life Science & Biotechnology, Korea University, Seoul 136-701, Republic of Korea

ARTICLE INFO

Article history:
Received 12 February 2013
Received in revised form 10 June 2013
Accepted 10 June 2013
Available online 28 June 2013

Keywords:
Rice bran
Immunostimulatory activity
Glycoprotein
Nitric oxide
Cytokines
RAW 264.7 cells

ABSTRACT

Many natural resources obtained from plants have been studied for their utility as host defense potentiators. In the present study, we investigated whether a glycoprotein fraction from rice (*Oryza sativa*) bran (GFRB) could modulate immune responses such as the production of nitric oxide (NO), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-10 in the RAW 264.7 macrophage cell line. GFRB, which contained 65.7% of protein and 7.7% of total sugar, was prepared by treating an aqueous extract of rice bran with 80% $(NH_4)_2SO_4$ and the extraction yield was 4.9%. GFRB consisted of 5 bands with varying molecular weights by SDS-PAGE and remarkably improved production of NO in RAW 264.7 murine macrophage cells, up to approximately 10-fold compared to the normal control at 100 μg/mL concentration. In RAW 264.7 cells treated with 50 μg/mL GFRB, released levels of various cytokines such as TNF-α, IL-1β, IL-6, and IL-10 were 2824.4 ± 90.7, 224.5 ± 4.0, 524.3 ± 4.8, and 143.0 ± 9.5 pg/mL, respectively, which were higher than the levels in normal controls. Moreover, GFRB exhibited no cytotoxicity. According to the results of region-selective enzyme hydrolysis, the immune responses against GFRB were elicited by the glycans in the GFRB. These results show the potential of GFRB as a functional therapeutic agent with demonstrable immunostimulatory activity.

1. Introduction

Oryza sativa L., commonly known as Asian rice, is the most important staple food in a large part of the world, especially in Asia. Global rice production is expected to reach 481 million metric tons (milled basis) in 2011, on the back off weather conditions [1]. Nearly all rice is milled before consumption and as a result approximately 7% rice bran was produced as a byproduct of the rice milling process [2]. As rice bran has not been consumed typically in an ordinary diet due to its poor mastication and possible hull contamination, most is used directly as an ingredient in animal feed. This undervalued byproduct of rice milling, however, is a rich dietary source of phytochemicals such as γ-oryzanol, ferulic acid, tocopherols, and tocotrienols, which have been reported as strong antioxidants [3].

Proteins extracted from rice bran have been recognized as nutritionally superior to proteins from other sources, particularly because of their reported hypoallergenicity [4] and anti-cancer activity [5]. Glycans of binding forms also exist in the protein extract of rice bran [6], and though the presence of glycans in plant protein extracts has been reported [7], little information is available on the binding of these glycans to the protein. Despite the recognized therapeutic potential of rice bran proteins, concentrates and isolates are not commercially available [8].

Modulation of the immune response to prevent diseases has long been a topic of interest to researchers [9]. Plant extracts have been studied widely for their potential therapeutic effects in immune-related functions. The immune system is a remarkably adaptive defense system against invading pathogens, and modulation of the regulatory mechanisms of the immune system has been studied for many years. The role and necessity of the immune system are prominent during chemotherapeutic intervention for the treatment of many diseases. In recent years, traditional herbal medicines such as echinacea, ginseng and astragalus have been investigated for their immunomodulatory potential as therapeutic agent against infections and neoplastic diseases [10]. Cancer patients may wish to take these herbal medicines to inhibit tumor growth or to boost resistance to infections. The results of an earlier report have shown that oral administration of rice bran is a promising dietary intervention to regulate mucosal immunity for protection against enteric infections and induction of beneficial gut bacteria [11]. Several active materials have also been isolated and characterized from rice bran [3], though their

* Corresponding author. Tel.: +82 31 780 9068; fax: +82 31 709 9876.
E-mail address: chdon@kfri.re.kr (H.-D. Choi).

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http://dx.doi.org/10.1016/j.intimp.2013.06.013

efficacy in modulating immune-related functions has not been investigated in detail.

Activated macrophages exhibit the ability to induce production of several cytokines, which perform critical functions in a variety of immune responses. Some cytokines such as tumor necrosis factor (TNF)-α and the members of the interleukin (IL) family are predominantly secreted. Therefore, in the present study, we characterized the glycoprotein fraction from rice bran (GFRB) and investigated its immunostimulatory activities in the mouse macrophage cell line RAW 264.7 by assessing proinflammatory cytokines such as TNF-α, IL-1β, IL-6 and nitric oxide (NO).

2. Materials and methods

2.1. Preparation of GFRB

Dried rice bran (100 g), with a particle size under 0.5 mm, was collected from the Kyungdong market (Seoul, Republic of Korea), grounded and defatted with 1 L *n*-hexane at 4 °C. The defatted rice bran flour was extracted according to methods reported by Cuadrado et al. [12], except using 20 mM Tris–HCl (pH 8.0) containing 10 g of polyvinylpyrrolidone as the extraction buffer. The solution was filtered using a 0.45 μm filter, and the crude aqueous extract was precipitated with 80% $(NH_4)_2SO_4$ and then dialyzed using a dialysis membrane (Spectra/por, MWCO 6000 8000, Spectrum Laboratory Inc., Rancho Dominguez, CA, USA) against 20 mM Tris–HCl (pH 7.4) at 4 °C overnight. After dialysis, the contained solution was lyophilized to yield the GFRB and stored at −70 °C.

2.2. Characterization of GFRB

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [13] by using 15% acrylamide slab gel. GFRB (100 mg) was loaded and the gel stained with Coomassie blue R-250. Molecular mass markers (7–240 kDa) of DokDo-MARK™ were purchased from ELPIS-Biotech (Daejeon, Republic of Korea).

The total sugar content of the GFRB was determined by the phenol-sulfuric method [14], using glucose as a standard. The correct amount of GFRB was dissolved in 200 μL deionized water (DW), mixed well with 200 μL 5% phenol, and then added to 1 mL of sulfuric acid. The mixture was incubated at room temperature for 20 min. The total sugar content was measured at 490 nm. Protein content of the GFRB was determined with the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA), using bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) as a standard. The GFRB solution (25 μL) was added to 200 μL of the BCA reagent and then incubated at 37 °C for 30 min. The standard and samples were measured spectrophotometrically at 540 nm.

Chromatographic conditions were modified from Burana-osot et al. [15]. To determine the monosaccharide compositions of GFRB, 1 mg of samples was dissolved in 1 mL DW, and an equal volume of 4 N HCl was added and allowed to stand for 4 h at 100 °C under gentle stirring. After the reaction, the mixture was filtered through a 0.45 μm syringe filter and vacuum-dried using a Speed-Vac (Module spin 40, Biotron, Korea). The vacuum drying was repeated 3 times to completely remove any residual HCl. Monosaccharide analysis of the hydrolysate was performed by high performance anion-exchange chromatography (HPAEC) using the Ion Chromatography System (ICS-3000, Dionex Co., USA) equipped with a pulsed amperometric detector (ED 50, Dionex Co., USA). The dried sample was dissolved in 0.1 mL DW and filtered through microspin filters (0.45 μm, PGC Scientifics, Frederick, MD). After filtration, 20 μL of the sample was injected and fractionated on a CarboPac PA-1 column (4 × 250 mm, Dionex Co., USA) which was pre-equilibrated with 200 mM NaOH. A sample containing monosaccharides was eluted isocratically with 10 mM NaOH for 25 min, followed by a linear gradient of 0 150 mM sodium acetate in 100 mM NaOH for 20 min to elute acidic monosaccharides. Before each injection, the column was re-equilibrated by running with 100 mM NaOH for 15 min and then with 10 mM NaOH for 10 min. The eluent flow rate was maintained constant at 0.5 mL/min.

The limulus amebocyte lysate (LAL) assay was used to estimate the amount of endotoxin in the GFRB. Analysis of endotoxin concentration was performed via the kinetic method (Pierce® LAL Chromogenic Endotoxin Quantitation Kit, Thermo Scientific, USA) using a microplate reader (EL 800, BioTek Instruments, Winooski, VT, USA).

2.3. N-glycan analysis

N-glycans were released from GFRB by successive treatments with hydrolase. In brief, 100 mg GFRB in 3 mL 0.05 M Tris–HCl (pH 8.0) was proteolyzed with trypsin (1 mg/mL) and chymotrypsin (1 mg/mL), and the proteolysate was further digested with 200 μU glycoamidase A followed by pronase (1 mg/mL) to release *N*-glycans. After removing the hydrolyzed materials with gel filtration by P-2 resin, the reducing ends of *N*-glycans were derivatized with 2-aminobenzamide (2-AB). Labeled *N*-glycans of GFRB were applied normal-phase HPLC system [16]. The glucose unit (GU) values of the separated *N*-glycans were determined from a retention time of a 2-AB labeled glucose homopolymer ladder (Ludger, Oxfordshire, UK). Glycan structures were determined from GU values compared to reference values (GlycoBase).

2.4. Cell culture, morphological observations and viability

RAW 264.7 cells were obtained from the Korean Cell Line Bank (Seoul, Republic of Korea) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco/BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco/BRL, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco/BRL, USA) at 37 °C in 5% CO_2. Mitochondrial respiration, an indicator of cell viability, was assessed by a mitochondrial-dependent reduction of a yellow tetrazolium dye 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) to insoluble purple formazan by dehydrogenases (Duchefa Biochemie, Haarlem, Netherland). Briefly, cells were seeded at a concentration of 2×10^5 cells/well in a 96-well plate (Nunc, Germany) and treated with varying doses of GFRB (12, 25 and 50 μg/mL). After incubation for 24 h, cells were observed by light microscopy using an Olympus CKX41 (Olympus Optical Co., Tokyo, Japan) at 200× magnification. After morphological observations, 20 μL of MTT stock solution (5 mg/mL, Sigma-Aldrich) was added to each well and the plates were further incubated for 3 h at 37 °C. The supernatant was removed and 100 μL of DMSO (Sigma-Aldrich) was added to each well to solubilize the water-insoluble purple formazan crystals. The absorbance at 570 nm was measured using a microplate reader.

2.5. Quantification of NO production

NO levels in the RAW 264.7 cells were determined by calculating the amount of released nitrite by using Griess reagent (Sigma-Aldrich) according to the Griess reaction. First, 2×10^5 RAW 264.7 cells/well were suspended in DMEM supplemented with 10% FBS and plated in 96-well culture plates. The cultures were incubated for 24 h at 37 °C in an atmosphere of 5% CO_2 and 95% humidity. To determine the NO concentration, 100 μL of the supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylenediamine dihydrochloride, and 0.5% H_3PO_4), and the mixture was kept at room temperature for 15 min. The absorbance at 540 nm was determined using a microplate reader. NO levels were estimated using a standard curve plotted against a known quantity of $NaNO_2$.