Immunomodulatory Effects of Nontoxic Glycoprotein Fraction Isolated from Rice Bran

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Abstract

Rice bran, a by-product of brown rice milling, is a rich source of dietary fiber and protein, and its usage as a functional food is expected to increase. In this study, immunomodulatory effects of glycoprotein obtained from rice bran were studied in normal mice and mouse models of cyclophosphamide-induced immunosuppression. We prepared glycoprotein from rice bran by using ammonium precipitation and anion chromatography techniques. Different doses of glycoprotein from rice bran (10, 25, and 50 mg/kg) were administered orally for 28 days. On day 21, cyclophosphamide at a dose of 100 mg/kg was administered intraperitoneally. Glycoprotein from rice bran showed a significant dose-dependent restoration of the spleen index and white blood cell count in the immunocompromised mice. Glycoprotein from rice bran affected the immunomodulatory function by inducing the proliferation of splenic lymphocytes, which produce potential T and B cells. Moreover, it prevented cyclophosphamide-induced damage of Th1-type immunomodulatory function through enhanced secretion of Th1-type cytokines (interferon-γ and interleukin-12). These results indicate that glycoprotein from rice bran significantly recovered cyclophosphamide-induced immunosuppression. Based on these data, it was concluded that glycoprotein from rice bran is a potent immunomodulator and can be developed to recover the immunity of immunocompromised individuals.

Abbreviations

ConA:	concanavalin A
GFRB:	glycoprotein fraction from rice bran
GRB:	glycoprotein from rice bran
IFN-γ:	interferon-γ
IL:	interleukin
LPS:	lipopolysaccharide
NO:	nitric oxide
PSK:	polysaccharide-K
Th (cells):	T helper (cells)
WBC:	white blood cell

Introduction

Exposure to environmental chemical carcinogens and typical lifestyle factors, including cigarette smoking, UV exposure, and severe stress, led to an increase in the cancer incidence rate [1,2]. Most of the anticancer drugs widely used in chemotherapy are known to cause severe adverse effects including immune system dysfunction, which may affect patients' quality of life in addition to the constraints imposed by tumor treatment [3]. Most chemotherapeutic agents (e.g., cyclophosphamide and cyclosporin A therapy) cause immunosuppression in patients. Cyclophosphamide is an alkylating agent widely used as an anticancer drug; it causes toxicity because of its reactive metabolites, such as acrolein and phosphoramide mustard. These toxic metabolites induce immune dysfunction or immunosuppression followed by hemorrhagic cystitis [4], hematopoietic depression [5], and cardiac damage [6]. Reducing undesirable side effects in immunosuppressed animal models may thus become beneficial to patients overcoming chemotherapy-associated toxicities. To reduce the toxicity and enhance the curative effect of chemotherapy, many researchers have developed immunomodulators that enhance host defense responses, which can be an effective way to increase resistance to aftereffects [7].

Approximately 60% of the therapeutic proteins in natural products are glycoproteins [8]. Researchers are interested in plant glycoproteins that have a broad spectrum of immunostimulatory activities and relatively low toxicity. These glycoproteins are known to improve immune function by stimulating specific immune-related cells such as phagocytes, lymphocytes, and natural killer cells, and also by promoting humoral immune responses [9]. Therefore, we extracted and characterized GFRB, investigated its immunostimulatory activity, and confirmed the effects of glycan on immune responses as reported in a previous study [10].

In the present study, we prepared GRB by isolation from GFRB and then evaluated its protective effects against cyclophosphamide-induced immunosuppression in mice. We also investigated the immunomodulatory effects of GRB by analyzing the mitogenic activities of splenocyte and plasma lysozyme activity.

Results

Ten fractions were isolated from GFRB by using a Q-sepharose anion-exchange resin column with gradient elution to investigate the effect on NO production by RAW 264.7 macrophage cells (**Fig. 1**). We measured the concentration of nitrite, the oxidative metabolite of NO, in cell culture medium by using the Griess reaction method. A minimal amount (under 5 µM) of NO was produced when RAW 264.7 cells were incubated with the medium alone, whereas treatment of these cells with three isolated fractions (F1, F2, and F9) resulted in a concentration-dependent increase in NO production, with fraction F1 being the most active (**Fig. 1 B**). Indeed, NO production induced by 100 µg/mL F1 was comparable to that induced by 1 µg/mL LPS. Moreover, the Q-sepharose unbound F1 fraction was considered a protein-bound glycan because its maximum absorbance peaks were obtained at 490 nm (phenol-sulfuric acid assay) and 280 nm (spectrophotometer); the fraction was named GRB (**Fig. 1 C**).

The gel was stained with a Coomassie blue stain to visualize the three major bands from GRB, spanning approximately 25–40 kDa (**Fig. 1 D**). We used GRB, comprising three different molecules (25, 35, and 40 kDa), and these molecules differ from the major allergen in rice, which shows protein bands of 14–16 kDa [11].

GRB, white powder, contains approximately 55.8% protein (determined using a BCA method with BSA as a reference protein) and 5.1% carbohydrate (determined using a phenol-sulfuric acid method with glucose as a reference sugar).

As shown in **Fig. 2**, polymyxin B almost completely suppressed NO production induced by LPS (LPS: 18.3 µM of NO versus LPS + polymyxin B: 2.4 µM of NO). In contrast, polymyxin B did not suppress responses induced by GRB (GRB: 29.3 µM of NO versus GRB + polymyxin B: 28.8 µM of NO). These results indicate that the observed immunomodulatory response of GRB is independent of any potential endotoxin contamination.

Immunomodulatory effects of GRB were evaluated in mouse models of cyclophosphamide-induced immunosuppression. The body weight gain of the normal and experimental mice throughout the experiment is shown in **Table 1**. There was no significant difference between the body weight gains of mice from the normal and experimental groups.

To assess the effect of GRB on immunocompetent organs, we analyzed the spleen index of mice. The spleen index in the cyclophosphamide group was found to be 3.67, which was significantly lower than that in the normal control group (4.53). The GRB50 group's spleen index (4.18) was significantly higher than that of the cyclophosphamide group, and was maintained at a similar level as that of the normal control group. Moreover, administration of 50 mg of GRB per mouse did not result in any difference in the spleen index compared to that of the normal control group.

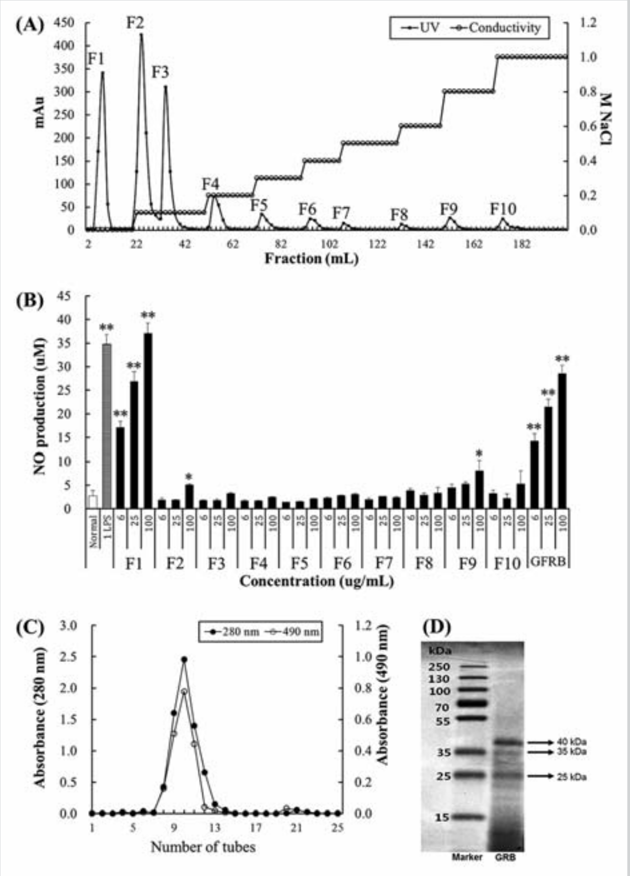

Fig. 1 Ten fractions were separated by glycoprotein fractionation from rice bran using anion exchange chromatography, and their effects on NO production in RAW 264.7 cells were observed. **A** Chromatogram of the F1–F10 fractions from a Q-sepharose FF column eluted with buffer (20 mM Tris-HCl, pH 9.0) and NaCl (0–1.0 M) at a flow rate of 1.0 mL/min. **B** *In vitro* activation of RAW 264.7 cells stimulated with different concentrations of the 10 fractions in terms of NO production. **C** Chromatogram of F1 from a Superdex-75 column (2 cm × 95 cm) eluted by 20 mM Tris-HCl (pH 9.0) at 0.1 mL/min. **D** SDS-PAGE analysis. The results are from three independent experiments and presented as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. normal control.

There was a significant reduction in the WBC count of mice treated with cyclophosphamide as an immune defense marker; however, the WBC counts recovered after administration of combination treatment with cyclophosphamide and GRB. As shown in **Fig. 3 A**, treatment with cyclophosphamide significantly decreased (76.4%) the WBC count compared to that in the normal group. GRB administration increased the WBC count in mice with cyclophosphamide-induced immunosuppression in a significant and dose-dependent manner. There was also a significant increase in the WBC count in normal mice after treatment with GRB (50 mg/kg) only.

Lysozyme has both bactericidal and phagocytic properties that activate the complement system and prevent infection, respectively [12]. In practice, lysosomal activity is determined by the rate of lysis of *Micrococcus lysodeikticus*. **Fig. 3 B** shows that sig-

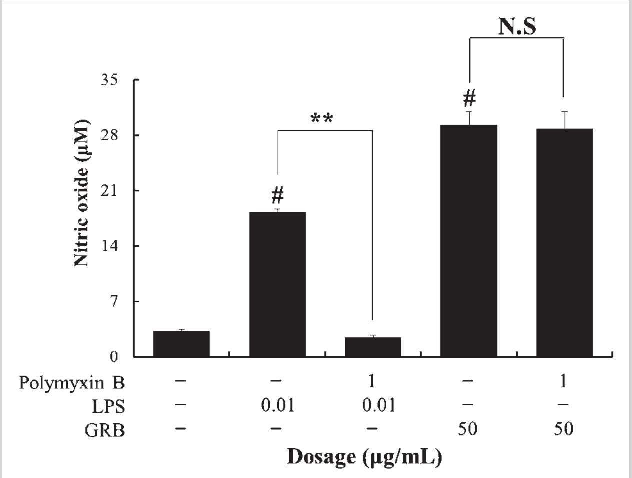

Fig. 2 Effect of a diet supplemented with GRB on NO production in RAW 264.7 macrophages in the presence or absence of polymyxin B (1 μg/mL). LPS was used as the positive control. The indicated GRB or LPS was preincubated with polymyxin B (1 μg/mL) for 1 h. RAW 264.7 cells were incubated for 24 h with untreated and polymyxin B-treated GRB (50 μg/mL) or LPS (1 μg/mL), and the cultured supernatant was collected to measure NO production with Griess reagent. Values are expressed as mean ± SEM of three independent experiments. $^{\#\#}P < 0.01$ vs. normal group, $^{**}p < 0.01$ vs. LPS-treated group (0.1 μg/mL).

nificantly higher levels of plasma lysozyme activity were detected in GRB-treated mice compared to those in mice with cyclophosphamide-induced immunosuppression. Moreover, significantly higher phagocytic activity was induced in only the 50 mg GRB-administered group.

Lymphocytes are the fundamental effector cells of immune responses. In the present study, effects of GRB on spleen lymphocyte proliferation were observed with stimulant mitogens (T cell mitogen ConA and B cell mitogen LPS) and without mitogens. As shown in ▶ **Fig. 4A**, after administration of GRB (50 mg/kg) alone for 4 weeks, the proliferation rate of mice splenocytes significantly increased compared to that of normal cells. In the absence of mitogen, administration of GRB at 25 and 50 mg/kg restored splenocyte proliferation significantly in mice with cyclophosphamide-induced immunosuppression. GRB (50 mg/kg) and PSK showed similar levels of splenocyte proliferation indices compared to those observed in the normal group without mitogen and LPS-induced splenocytes. In addition, a significantly increased proliferation index was observed in the ConA-induced GRB (50 mg/kg) and PSK groups.

In ▶ **Fig. 4B, C**, intraperitoneal treatment with cyclophosphamide caused a significant reduction in IFN-γ and IL-12 levels (35.6% and 20.9% versus the normal control group, respectively). In contrast, in the animals treated with cyclophosphamide along with GRB (25 and 50 mg/kg), these parameters increased in a dose-dependent manner. PSK, a positive control, also significantly increased the levels of these cytokines. In addition, administration of 50 mg GRB per kg mouse for 28 days had no significant effect on IFN-γ and IL-12 levels.

Table 1 Effects of GRB on body weight gain and spleen index in cyclophosphamide-treated immunocompromised BALB/c mice.

Dosage (mg/kg mouse)	Body weight gain (g)	Spleen index[1]
Normal	1.10 ± 0.54^a	4.53 ± 0.32^a
Cyclophosphamide (100)	0.63 ± 0.39^a	3.67 ± 0.18^b
PSK (25) + cyclophosphamide (100)	1.29 ± 0.51^a	3.60 ± 0.21^b
GRB (10) + cyclophosphamide (100)	0.86 ± 0.32^a	3.57 ± 0.15^b
GRB (25) + cyclophosphamide (100)	1.24 ± 0.43^a	$4.06 \pm 0.16^{a,b}$
GRB (50) + cyclophosphamide (100)	1.39 ± 0.61^a	4.18 ± 0.29^a
GRB (50)	1.35 ± 0.40^a	4.56 ± 0.16^a

[1] Spleen index, mg spleen per g body weight; ^{a,b} Means with different letters are significantly different ($p < 0.05$) by Duncan's multiple range test

Discussion

Endotoxins such as LPS function as an immunomodulator and are often present as contaminants in biological preparations [13]. Since the possibility of LPS contamination in the GRB exists, we evaluated the effects of polymyxin B on macrophage NO production induced by GRB. Polymyxin B can bind to the lipid A region of LPS and inhibit its activity [14]. However, polymyxin B was unable to inhibit the stimulatory effect of GRB on NO production, while inhibiting the positive LPS control. The results showed that LPS was blocked by polymyxin B, while GRB did not change the effect. Therefore, we are confident that the immunomodulatory activity in GRB is not a contaminating artifact.

Cyclophosphamide is one of the most commonly used anticancer and immunodepressant drugs used for preventing graft rejection, treating chronic autoimmune diseases, and inducing experimental immunosuppression [15]. It can inhibit the proliferation of cancer cells by sticking to DNA strands and can inhibit both humoral and cellular immunity as well [16]. Damage to the immune system is one of the major side effects of chemotherapy. Potential cytotoxic drugs such as cyclophosphamide act on the cells of the immune system at various levels [15, 17, 18]. Destruction of T and immune-related cells as well as innate immune responses is the major drawback of cyclophosphamide therapy.

Our previous study indicated that the glycan moiety of GFRB was responsible for its immunomodulatory activity [10]. The mechanism of action of GRB in terms of stimulation of both the cellular and the humoral immunity is not simple. In the present study, GRB was isolated from GFRB by using anion exchange chromatography and its immunomodulatory activity was studied in an animal model of cyclophosphamide-induced immunosuppression. Decreased immune function parameters such as WBC counts, lysozyme activity, lymphocyte proliferation, and cytokine production in the cyclophosphamide-treated group compared to the parameters in the vehicle-treated normal control group indicated that an immunosuppressed condition was well established in our experimental animal model. PSK was used as a positive control. It is a widely used mushroom (*Coriolus versicolor*) extract consisting of glycoproteins and has shown antitumor and immunomodulatory effects in both preclinical and clinical studies [19, 20].

WBC count is a frequently studied clinical parameter that well reflects a chemotherapeutic disorder [21]. Spleen index, the ratio between spleen and body weight, is also used as indicator of immunosuppression. A high spleen index indicates the presence of an infection and/or inflammatory condition. On the contrary, a

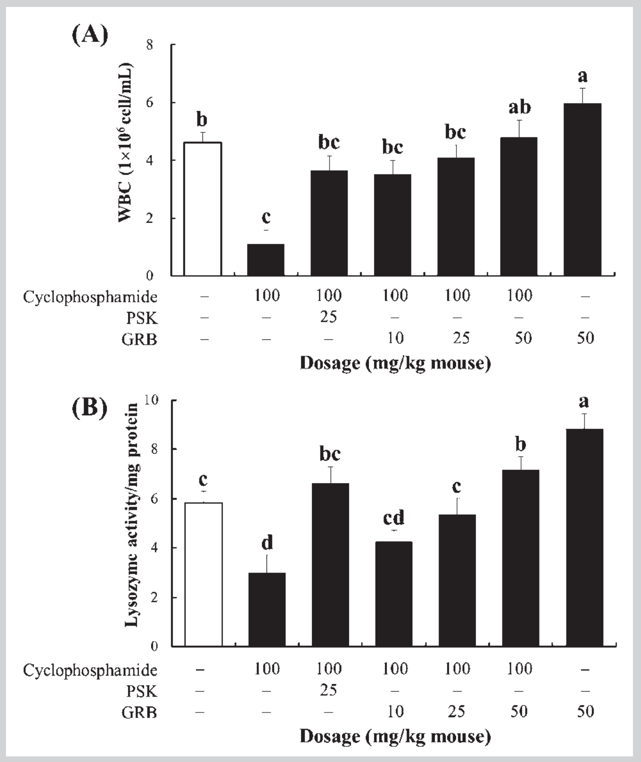

Fig. 3 Effect of diet supplemented with GRB on WBC count and plasma lysozyme activity in normal and cyclophosphamide-induced immunosuppressed BALB/c mice. $^{a–d}$ Means with different letters are significantly different ($p < 0.05$) by Duncan's multiple range test.

low spleen index indicates immunodeficiency. The spleen plays an important role in the immune system, as the white pulp is composed of B lymphocyte-rich lymphoid follicles and T lymphocyte-rich periarteriolar lymphoid sheaths. Cyclophosphamide reduced the spleen index and WBC count to the lowest level about 7 days after administration in our preparatory experiment (data not shown). Therefore, we administered 100 mg of cyclophosphamide per kg body weight/mouse to the animals, and evaluated their immune status on the basis of their WBC counts (▶ **Fig. 3A**) and spleen indices (▶ **Table 1**). GRB significantly restored the WBC count in a dose-dependent manner, suggesting that it could provide preferential protection against leukopenia induced by cyclophosphamide. The WBC count in the cyclophosphamide group was significantly different from that of the group receiving GRB for 4 weeks. Administration of GRB also ameliorated the decrease in the spleen index in immunosuppressed mice. However, there was no effect on the normal spleen index, showing that oral administration of < 50 mg/kg GRB does not cause hyperimmunization. These results indicate that GRB restores immune cell production that is damaged by cyclophosphamide, and also shows that GRB does not affect non-immunoreactive organs. Thus, these findings indicate that GRB could enhance patients' ability to tolerate immune system disorders caused by cyclophosphamide treatment.

In mammalian cells, phagocytosis is an essential defense mechanism that protects cells against pathogen invasion. Phagocytes like granulocytes, macrophages, dendritic cells, and mast cells perform the function of scavenging debris [22]. Phagocytosis is performed either within the phagocyte (e.g., respiratory burst) or outside of the phagocyte (e.g., cytokines, lysozymes, lactofferins, and proteases). Phagocytosis via lysozyme activity plays an important role in innate defense mechanisms against ingested foreign materials. Phagocytosis involves specialized plasma lysozyme activity. In this study, a dose-dependent increase in *M. lysodeikticus* lysis was observed, which may be due to increased lysozyme activity by GRB (▶ **Fig. 3B**). These data reveal that GRB can elevate phagocytic activity both *in vitro* and *in vivo*. Proliferation of lymphocytes following exposure to mitogenic stimuli is an important indicator of improvement in cell-mediated immunity of T or B lymphocytes, which is a typical nonspecific immune response with a well-understood mechanism. Moreover, because of its high sensitivity, this methodology has been widely used as an immune parameter to investigate lymphocyte responsiveness [23]. Our results showed that the experimental groups administered GRB displayed a dose-dependent recovery of splenocyte proliferative responses to both T and B lymphocytes in cyclophosphamide-treated mice (▶ **Fig. 4A**). GRB administration caused a mitogenic effect similar to that of ConA. However, GRB administration had a much weaker influence on B lymphocyte proliferation than on T lymphocyte proliferation. Different mechanisms might be responsible for the effect of GRB administration on the proliferation of B lymphocytes. The increased population of CD4$^+$ T lymphocytes further indicated that Th cells were activated by GRB administration. Th cells are generally classified into four subsets, namely, Th1, Th2, Th17, and regulatory T (Treg) cells, which have different cytokine production abilities [24].

Two types of Th cells (Th1 and Th2) regulate the development of antibody-mediated humoral and T cell-mediated cellular immunity. The differential immune pathways are mediated by two types of Th cells that produce distinct immunoregulatory cytokines. The production of IFN-γ, IL-12, and IL-2 triggers Th1-type cellular responses, while the production of IL-4 and IL-5 triggers Th2 cellular responses. These cytokines can directly or indirectly regulate immune reactions [25]. In the present study, we demonstrated that GRB administration restored the Th1-related cytokines, IL-12 and IFN-γ, in the splenocytes of cyclophosphamide-induced immunosuppressed mice (▶ **Fig. 4B, C**), while it had no effect on the Th2-related cytokines IL-4 and IL-5 (data not shown). Thus, Th1 cells might be the main target cells of GRB in the immunosuppressed model induced by cyclophosphamide. Since the levels of Th2 cytokines are higher than those of Th1 cytokines in immune-related diseases including allergies and asthma [26], these results also suggested that GRB might be useful as an agent for Th2 dominant pathological disorders.

In summary, the results from our cyclophosphamide-induced immunosuppressed mouse model orally administered GRB for 4 weeks suggest that GRB has an immunomodulatory activity. Although the exact underlying mechanism of this activity is unknown, based on the results presented above, we conclude that the immunomodulatory effect of GRB is related to the enhancement of the host immune system function, which might mainly be caused by GRB-mediated activation of T lymphocytes and macrophages and the stimulated secretion of specific cytokines.

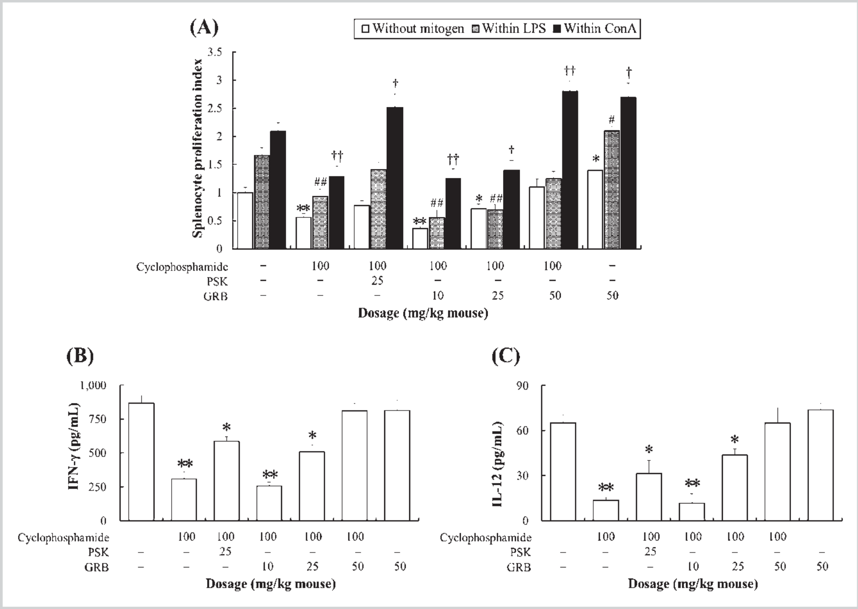

Fig. 4 Effect of diet supplemented with GRB on splenocyte isolated from cyclophosphamide-treated immunosuppressed BALB/c mice. **A** Splenocyte proliferation was observed with stimulant mitogens such as ConA, LPS, or without mitogens. IFN-γ (**B**) and IL-12 (**C**) levels were determined using supernatant without mitogen cells. Data are expressed as the mean ± SD of the results for six mice. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. mitogen-untreated normal control group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. LPS-induced normal group; $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ vs. ConA-induced normal group.

Materials and Methods

▼

Preparation of glycoprotein from rice bran

Milled rice bran was collected from Kyungdong market in Seoul, South Korea, and GFRB was extracted from it as described previously [10]. For further isolation of glycoprotein, the sample was applied to an anion exchange Q-sepharose fast flow column (Atoll GmbH). Three-milliliter fractions were collected and the absorbance of the protein was monitored at 280 nm. Ten peaked fractions (▶ **Fig. 1 A**) were lyophilized after dialysis against deionized water for 24 h at 4 °C. The fraction that showed the highest NO production activity with RAW 264.7 cells was referred to as GRB. In addition, GRB was applied to a Superdex-75 column (2 cm × 95 cm), and eluted with 20 mM Tris-HCl buffer (pH 9.0) at a flow rate of 0.1 mL/min. Three-milliliter fractions were collected and the absorbance values at 280 nm for protein and 490 nm for carbohydrate were monitored.

Determination of nitric oxide production

RAW 264.7 macrophage cells obtained from the Korean Cell Line Bank were cultured in phenol red free Dulbecco's modified Eagle's medium (Gibco/BRL). NO levels in the RAW 264.7 cells were determined by calculating the amount of released nitrite using Griess reagent (Sigma-Aldrich) according to Griess's reaction.

Total carbohydrate and protein analysis

The carbohydrate content of the GRB was determined using the phenol-sulfuric acid reaction method, with glucose as a reference sugar and absorbance measured at 490 nm using a microplate reader. To determine the protein content of the GRB, the BCA method was used, with BSA as the standard. The absorbance value at 280 nm was also used to determine the protein content of GRB.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method described by Laemmli [27] by using a 15% acrylamide slab gel. GRB (80 mg) was loaded and the gel was stained with Coomassie blue R-250. Molecular mass markers (15–250 kDa) of DokDo-MARK™ were purchased from ELPIS-Biotech.

Determination of endotoxin contamination

Because LPS induces NO production from macrophages, we assessed the roles of LPS or LPS-like activity in GRB. Possible endotoxin contamination was monitored by determining polymyxin B sensitivity. Polymyxin B, a peptide antibiotic, neutralizes LPS through binding the lipid A moiety of the endotoxins. To determine the sensitivity to polymyxin B, RAW 264.7 cells were cultured with GRB (50 µg/mL) or LPS (1 µg/mL; Sigma-Aldrich) from *Escherichia coli* 055:B5 in the presence or absence of polymyxin B (1 µg/mL; Sigma-Aldrich) for 24 h, and NO levels in the cell-free supernatants were determined. Results were calculated as endotoxin units (EU) per mL of dried sample.

Animals

Before being fed, specific pathogen-free 6-week-old female BALB/c mice (Samtako Bio) weighing 19–21 g were acclimatized for 1 week in the animal experimental research laboratory and randomly divided into seven groups with 10 mice per group. Mice were housed in an air-conditioned room at 22 ± 2 °C with a relative humidity of 55 ± 5% under a 12-h light/dark cycle and were fed sterilized filtered water and a standard laboratory diet (AIN-93G diet, Dyets). The experimental protocols of the study were in compliance with the Korea Food Research Institutional Animal Care and Use Committee (Receive number, 2012–0041, approved on August 31, 2012; approval number, KFRI-M-13 005) regarding technical specifications for the production, care, and use of laboratory animals.

GRB for oral administration was freshly prepared as a homogenized suspension at doses of 10, 25, and 50 mg/kg each in 0.9% saline, and administered orally once daily to the mice for the duration of the experiment (28 days). PSK (667 mg/g, obtained from Kwang Dong Pharmaceuticals Co.) at a dose of 25 mg/kg was used as the positive control. Cyclophosphamide was used as the immunosuppressive agent at 100 mg/kg by intraperitoneal injection on day 21.

Total and different leukocyte count

Collected blood samples were diluted with Turk's solution (Merck) in a WBC pipette, in which red cells were lysed without affecting the leukocyte and observed using a flow cytometric analyzer (XE-2100D, Sysmex Co.).

Plasma lysozyme assay

Plasma samples were extracted from the experimental mice and centrifuged at 1500 × *g* for 10 min. Lysozyme activity was determined with a minor modification to the method previously described by Nudo and Catap [28], which measures the lysis of *M. lysodeikticus* (Sigma-Aldrich).

Splenocyte proliferation and mitogenic activity assays

Mitogenic activity was tested using a slightly modified method described previously [26]. For splenic T lymphocytes, we conventionally employed plant lectin (ConA), whereas splenic B lymphocytes were stimulated by bacterial lectin (LPS). In the mitogenic test, ConA (4 µg/mL) or LPS (2 µg/mL) was added 24 h after splenocyte culturing, and further incubated for 48 h. Then, splenocyte proliferation and mitogenic activity were assayed by the MTT method.

Quantification of interferon-γ and interleukin-12 production in splenocytes

After incubation of the isolated splenocytes, as described in the previous section 2.4.6., the culture medium was collected, filtered (0.45 mm), and analyzed for the presence of IFN-γ and IL-12. Levels of individual cytokines were estimated by a sandwich ELISA kit (Enzo Life Science) according to the procedure described by the manufacturer.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Statistical evaluations between sample mean values were performed using Duncan's multiple range test and one-way analysis of variance (ANOVA) using the PASW Statistics 18 software (SPSS, Inc.). The minimal level of significance was set at $p < 0.05$.

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Conflict of Interest

The authors declare no conflict of interest.

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