Food and Chemical Toxicology 49 (2011) 910–916

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

Toxicological and immunomodulatory assessments of botryosphaeran (β-glucan) produced by *Botryosphaeria rhodina* RCYU 30101

Brian Bor-Chun Weng [a], Yu-Chih Lin [b], Chia-Wen Hu [a], Ming-Yuan Kao [b], Shih-Hao Wang [b], Dan-Yuan Lo [c], Tzu-Yuan Lai [b], Lou-Sing Kan [d], Robin Yih-Yuan Chiou [b,*]

[a] Department of Microbiology, Immunology and Biopharmaceuticals, National Chiayi University, Chiayi 60004, Taiwan
[b] Department of Food Science, National Chiayi University, Chiayi 60004, Taiwan
[c] Department of Veterinary Medicine, National Chiayi University, Chiayi 60004, Taiwan
[d] Institute of Chemistry, Academia Sinica, Taipei 11571, Taiwan

ARTICLE INFO

Article history:
Received 17 February 2010
Accepted 2 October 2010
Available online 24 December 2010

Keywords:
β-Glucan
Botryosphaeran
Gel permeation chromatography
Sprague–Dawley rat
Immunomodulation
Nitric oxide

ABSTRACT

Toxicological and immunomodulatory activities of botryosphaeran (BR), a newly emerged β-glucan that comprises a β-(1→3) backbone and β-(1→6) branched glucose residues were assessed. BR was 1.82×10^6 Da (M.W.) estimated by reversely-linear equation constructed by regression of logarithms of standard polysaccharides and their retention times of gel permeation chromatography. Sprague–Dawley rats were daily gavage-administered with BR at doses of 0, 1.25, 12.5, and 125 mg/kg body weight (BW) for 28 d. Serum hematological and biochemical analysis of all treatment were all within normal ranges. Mitogen-stimulated lymphoblastogenesis of spleno-lymphocytes was enhanced by BR at doses of 1.25 and 12.5 mg/kg BW. Through *in vitro* comparative assessments, RAW 264.7 macrophage (RAW) cells were treated with BR and two commercial β-glucans, zymosan (ZY) and barley β-glucan (GB), to characterize their relative immunomodulatory properties. All three β-glucans stimulated phagocytosis on fluorescence-labeled *Escherichia coli*. At dose levels from 5 to 200 μg/mL for 24 h, nitric oxide produced by BR- and ZY-treated cells were higher than those produced by GB-treated and control groups. BR, ZY but GB also stimulated RAW cells in producing TNF-α. The results demonstrate that BR is toxicologically accepted and features as a potent immunomodulator.

1. Introduction

Various β-glucans have been studied, and many exhibit bioactivities and confer health benefits (Lowry et al., 2005; Volman et al., 2008). With a view to develop β-glucan-containing value-added products, exploration of new sources of β-glucans and characterization of their origin-dependent bioactivities are of importance. Among the β-glucans of fungal polysaccharides, comprising intracellular-, structural-, and exocellular-polysaccharides (EPS), fungal EPS' are most favored by manufacturers, mainly due to its ease of recovery. Recently, a fungal EPS derived from *Botryosphaeria rhodina*, botryosphaeran (BR), has been elucidated to be a homologous β(1→3) (1→6) glucan with a molecular weight up to one million Daltons (Barbosa et al., 2003).

The toxicological and immunomodulatory activities of BR have been meagerly investigated. Recently, antimutagenic, hypoglycemic, hypocholesterolaemic, and anticlastogenic activities of BR have been reported (Miranda et al., 2007, 2008). Moreover, botryosphaeran modified by sulfonation has been reported to be effective in inducing anticoagulant activity (Mendes et al., 2009). With regard to immunomodulatory assessments of other β-glucans from fungal origins, zymosan (ZY), derived from *Saccharomyces cerevisiae*, is one of the most investigated β-(1,3)-glucans [β-(1,3) backbone with a β (1→6) branched glucan]. It has been demonstrated that ZY is an activator of immune cells through its capacity to enhance production of immune mediators, such as reactive oxygen species, nitric oxide (NO), TNF-α, and IL-10 (Goldman et al., 1994; Ohno et al., 1996; Ljungman et al., 1998; Du et al., 2006). Generation of such immune mediators by β-glucan-primed macrophages is linked with their antimicrobial and antitumor activities. In addition, EPS from a liquid culture of *Leninus edodes* was reported to be involved in up-regulation of RAW 264.7 macrophages, enhancement of phagocytosis and NO synthesis, as well as increases in mRNA levels of TNF-α, IL-12, and IL-23 (Lee et al., 2008). It has also been suggested that the immunomodulatory characteristics of β-glucans are mainly dependent on their unique molecular structures in activation of varied glucan receptors (Brown et al., 2003).

Barley β-glucan (GB), a cereal-origin β-glucan is mainly composed of a β (1→4) back bone with a β-(1,3) branched glucan with no continuous 1,3-linkage (Tada et al., 2008). Dietary GB has been

* Corresponding author. Tel.: +886 5 2717613; fax: +886 5 2775524.
E-mail address: rychiou@mail.ncyu.edu.tw (R.Y.-Y. Chiou).

0278-6915/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fct.2010.10.036

effective in lowering serum cholesterol in rats (Kalra and Jood, 2000), and recently, dose-dependent activation of peritoneal macrophages by GB in order to enhance IL-6 and IL-8 production has been demonstrated (Tada et al., 2009).

These reports indicate that BR, ZY, and GB have unique structure-bioactivity characteristics and that all are potential candidates for use in comparative investigations of their immunomodulatory activities. Such studies may form the bases for subsequent β-glucan-related product development. In the first part of this study, in addition to molecular weight estimation by gel permeation chromatographic (GPC) analysis of BR, ZY, and GB, Sprague–Dawley rats were subjected to BR treatment in an *in vivo* experiment to address toxicology and immunity issues. In the second part, *in vitro* immune-assessments in RAW 264.7 macrophage cells were performed with BR, as well as with commercially available ZY and GB.

2. Materials and methods

2.1. Botryosphaeran (BR) preparation

2.1.1. Culture of *B. rhodina*

A molecularly identified culture of *B. rhodina* RCYU 30101 strain was used as a starter for the production of BR. For submerged cultivation, a basal medium with sucrose as a carbon source (containing $NaNO_3$, 3.0 g; KH_2PO_4, 1.0 g; $MgSO_4$ 7 H_2O, 0.5 g; KCl, 0.5 g; yeast extract, 1.0 g; and sucrose, 60 g per liter of deionized water) was used as the cultivation medium after sterilization at 121 °C for 15 min (Selbmann et al., 2003). After spreading the culture on a yeast malt agar plate and cultivating for 1 d, a single colony with an approximate diameter of 1 cm was aseptically removed and inoculated into a 500 mL Erlenmeyer shaking-flask (with a baffle base) containing 200 mL of cultivation medium. The flasks were shaken-cultivated at 30 °C on an orbital shaker at 150 r/min for 72 h. Then, 10 mL of the mycelial suspension was aseptically withdrawn and inoculated into a 500 mL Erlenmeyer shaking-flask containing 200 mL of cultivation medium and cultivated as above for 24 h. From that flask a series of 10 mL aliquots of the suspensions was withdrawn and inoculated into a series of 500 mL Erlenmeyer shaking-flasks, each containing 200 mL of cultivation medium, and cultivated as above for 36 h.

2.1.2. Purification of botryosphearan (BR) and molecular weight estimation

That 36 h cultivated broth was heated to 85 °C on a hotplate with mild stirring in inactivating mycelia and enhancing polysaccharide release. Then, the broth was filtered through four layers of filtering-cloth to collect crude broth. Subsequently, the crude broth was frozen in a freezer and, when needed, thawed under ambient temperature. The jelly-like coagulant formed in the broth after complete thawing was collected, lyophilized, and used in the *in vivo* experiment as crude BR.

For purification of crude BR for use in *in vitro* experiments, previously reported procedures (Barbosa et al., 2003; Selbmann et al., 2003; Crognale et al., 2006) were followed with modification. Briefly, the crude BR broth was blended with two volumes of 95% ethanol to form a jelly-like coagulant. That coagulant was collected and its volume was mixed with an equal volume of deionized water. The mixture was heated on a hot plate to 50 °C and ultrasonicated (Misonix Sonicator XL-2015, Heat Systems Inc., Farmingdale, NY) to prepare a transparent solution. The solution was filtered through four layers of filtering-cloth. The filtrate volume was then blended with two volumes of 95% ethanol to form a jelly-like coagulant. The procedure of 1:2 volume blending and filtering was repeated two more times. The final transparent solution was lyophilized to prepare dry BR for use in the *in vitro* immunomodulatory characterization experiments. The yield of dry BR was *ca.* 1.5 g/L broth. Aliquots (100 mg) of the sample were subjected to nitrogen (N) determination by the micro-Kjeldahl method. Additionally, carbon (C), hydrogen (H), nitrogen (N), and sulfur (S) levels were determined by element analyzer (Jeol JSX-3202 M, Jeol Ltd., Tokyo, Japan). The N content determined by the Kjeldahl method was 0.51 ± 0.02%, while the C, H, N, and S contents determined by element analyzer were 36.9 ± 0.1%, 6.90 ± 0.01%, 0.52 ± 0.01%, and 0%, respectively. Crude protein content was estimated ca. 3.2% (Kjeldahl N% × 6.25).

In accurate estimation of BR molecular weight by GPC, the procedure and column reported by Wu et al. (2009) was referred with modifications. A GPC system was equipped with an HPLC pump (L-2130, Hitachi Co., Ltd., Tokyo, Japan), a GPC column (Shodex OHpak SB-806M HQ, Showa Denko K. K. Chemicals, Kanagawa, Japan) (8.0 × 300 mm), a column oven, and an evaporative light-scattering detector (LSD, Sedex 75, Sedere Inc., Lawrencedville, NJ). Deionized water was used as mobile phase and also used in sample preparation. The column and detector temperatures were 60 °C and 50 °C and the flow rate and injection volume was 1.0 mL/min and 20 μL, respectively.

For constructing a referenced curve in estimation of molecular weights, blue dextran (Amersham Pharmacia Biotech UK Ltd., London, UK) with M.W. 2.0×10^6 Da and Shodex Standard P-82 (pullulan) (Showa Denko K.K. Shodex Group) comprising a series of standard pullulans with 805, 21.7, 10.0 and 6.0×10^3 Da of molecular weight. Each of standards, 100 ppm prepared in deionized water, was run separately and the resulting elution times in accordance with logarithms of molecular weights were applied for regression in construction of a standard curve. For molecular weight estimation, the purified BR, ZY, and GB were respectively weighed and homogenized with deionized water by ultra-sonication to prepare β-glucan solutions (100 ppm). Each solution was subjected to GPC analysis as that described above. Each molecular weight was accordingly estimated referring to the linear equation primarily constructed by retention times and logarithms of standards.

2.2. In vivo immunotoxicological study

2.2.1. Experimental animals

According to the guideline of the Toxicological Principles for the Safety Assessment of Food Ingredients, 2000, we conducted a 28-days long short-term toxicity study but only on single gender animals, each treatment of 10 male Sprague–Dawley rats were randomly assigned to four treatment groups subjected to gavage-administration of crude BR at doses of 0 (control), 31.25 (low), 312.5 (middle), and 3125 (high) mg/kg body weight (BW); dose levels that are equivalent to 0, 1.25, 12.5, and 125 mg/kg BW of purified BR, respectively. The basis of the dosage used in the current study was referred to Miranda et al. (2008) who gave botryosphaeran from 7.5 to 30 mg/kg for 15 d showed no genotoxicity. And the current high dose level of 125 mg/kg/BW was 10 times of the speculated effective dosage level which was mainly for toxicity examination consideration. Animals were housed in a temperature- and photoperiod-controlled environment (22–25 °C and 12 h light: 12 h dark, respectively). Crude BR that had been previously prepared as a colloidal solution by mixing a unit weight of crude BR with five volumes (w/v) of deionized water was administered daily and feeding regime was *ad libitum* on commercial rodent chow (Labdiet®, Richmond, VA, USA). Following treatment, the animals underwent pathological evaluation for evidence of toxicity after being euthanized with carbon dioxide. Blood and spleen samples were prepared, as described later in Sections 2.2.2. and 2.2.3, respectively, and subjected to serum and blood cell analyses and characterizations of immune functions. All uses of these animals for experimental purposes complied with the regulations of the Institutional Animal Care and Use Committee, National Chiayi University, Chiayi, Taiwan, ROC.

2.2.2. Hematology and blood biochemical analyses

From each rat, blood from a heart puncture was collected into an EDTA tube (K2 EDTA syringes, BD Vacutainer, Franklin Lakes, NJ, USA). A small aliquot of blood was subjected to complete blood-cell enumeration using a Hemavet® analyzer (CDC Technology, Inc., Irvine, CA, USA). Blood biochemical analyses were performed using a photometric colorimetric method with kits on a blood autoanalyzer (Roche® Cobas Mira Plus™, F. Hoffman-La Roche Ltd., Basel, Switzerland). All quantitative determinations were calibrated and adjusted to be in the ranges of established limits by Sharp (1998).

2.2.3. Assessment of lymphoblastogenesis

For lymphoblastogenesis evaluation, splenocytes in RPMI-1640 medium were layered onto Histopaque® (density 1.077; Sigma Chemical Co.) and then centrifuged at 300*g* for 30 min to obtain a lymphocyte layer. That layer was removed and washed twice with fresh phosphate buffer saline (PBS). The number of lymphocytes were determined, and the cell number was diluted to 1×10^6 /mL then seeded onto 96-well plates. Specific mitogens including concanavalin A (Con A, 10 μg/mL; Sigma Chemical Co.), lipopolysaccharide (LPS, 10 μg/mL; Sigma Chemical Co.), and phorbol 12-myristate 13-acetate (PMA, 50 ng/mL; Sigma Chemical Co.) plus ionomycin (ION, 250 ng/mL; Sigma Chemical Co.) were introduced to stimulate specific lymphoblastogenesis. Alamar Blue™ (Serotec Co., Oxford, UK) was added at 24 h intervals for the entire 72 h of cell cultivation. Specific absorbency was obtained by measuring the reduced form excited at 528/25 nm and the oxidized form emitted at 585/30 nm using a multi-detection microplate reader (FLX800, Bio-Tek Instruments, Inc., Winooski, VT). The level of lymphoblastogenesis was based on the difference of absorbency (Δ specific absorbency) between un-stimulated cell medium and mitogen-stimulated cell medium using mean absorbencies of triplicate measures.

2.3. In vitro immune functions assessments

2.3.1. Culture of RAW 264.7 macrophage cells and preparation of β-glucans

The RAW 264.7 murine macrophage cell line used in this study was obtained from BCRC. Unless stated otherwise, the cells were cultured in Dulbecco's Modified Eagle's medium containing 2 mM L-glutamine and 10% fetal bovine serum (Sigma Chemical Co.). The aforementioned purified BR was weighed and homogenized with PBS using an ultrasonic homogenizer to prepare a colloidal solution. That solution was dispensed into a series of microcentrifuge tubes and stored frozen (−20 °C) until required. Prior to introduction of BR to each immune-activity assay, the colloidal solutions were thawed, vortexed, and sonicated for 3 min to ensure solution consistency. For comparative purposes, ZY from *S. cerevisiae* and GB from barley (both Sigma Chemical Co.) were prepared similarly as that for BR and assessed concurrently.

2.3.2. Phagocytic activity

The procedure reported by Lehmann et al. (2000) for determination of phagocytosic activity through flow cytometry was followed with modification. Briefly, RAW 264.7 cells were pre-cultured in 24-well plate with the basal media supplemented with either BR, ZY, or GB (100 μg/10^6 cells) at 37 °C and in a 5% CO_2 humidified atmosphere for 24 h. After incubation, the culture supernatants were decanted, washed, and replenished with warm PBS. At an effector:target ratio of 1:10, fluorescence-stained *Escherichia coli* cells were inoculated into each plate well and the well contents immediately subjected to centrifugation at 50*g* for 5 min to enhance interaction between effector and target cells. After incubation at 37 °C and in a 5% CO_2 humidified atmosphere for an additional 3 h to allow phagocytosis to progress, the macrophage cells were trypsinized and removed for quantification of phagocytosis by flow cytometry. Data analysis was completed using CellQuest software (Becton Dickson).

2.3.3. Nitric oxide (NO) production

RAW 264.7 cells were seeded into each well of a 96-well plate at a density of 1×10^6/mL. Then, BR, ZY, or GB were deposited into separate series of wells at doses of 0, 5, 50, 100, and 200 μg/mL before further incubation at 37 °C and in 5% CO_2 atmosphere for 24 h and 48 h. LPS (Sigma Chemical Co.) was introduced to the wells at 50 ng/mL as a positive control. After incubation, 50 μL of supernatant was withdrawn and transferred into another 96 well-plate for nitrite determination by reaction with Griess reagent (Promega Co., Madison, WI). To each well, 50 μL of 1% sulphanilamide and 50 μL of 0.1% naphthylethene diamine hydrochloride were added and incubated in darkness at ambient temperature for 15 min. Absorbance at 550 nm of the reactants was analyzed using a 96 well plate reader (Bio-Tek Instruments, Inc., Winooski, VT). A standard curve was constructed by preparing a series of sodium nitrite solutions from 1.56 to 200 μM and subjecting them to quantification by the same procedure.

2.3.4. iNOS mRNA expression

RAW 264.7 cells (1×10^6/mL) were seeded into a series of wells of a 24-well plate and BR, ZY, or GB was deposited in separate wells at doses of 50, 100, and 200 μg/mL. The seeded plates were cultured at 37 °C under a 5% CO_2 atmosphere for 24 h. Total RNA was extracted using Trizol reagent following the manufacturer's instructions (Promega Co.) and RNA was reverse transcribed using a cDNA synthesis kit (Promega Co.). The synthesized cDNA was then amplified with Taq polymerase and specific primers for iNOS and GAPDH (Promega Co) in a thermal cycler. Optimized conditions for RT-PCR were 60 °C for 30 s and 56 °C for 1 min for 30 cycles for both iNOS and GAPDH, and a final extension was conducted with denaturation at 94 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. Each 25 μL of reaction mixture contained 0.5 μM of each primer, 100 μM of dNTPs, and 1 U Taq DNA polymerase. The primers were 5′-GTCAACTGCAAGAGAACGGAGAAC-3′(sense) and 5′-GAGCTCCTCCAGAGGGTAG GCT-3′(antisense) for iNOS; and 5′-CAGAGCTCCAATCAACTGTCG-3′(sense) and 5′-TAAGTTAGATCCAGCTCCGACT-3′(antisense) for GAPDH. A total 25 μL of the final PCR products were analyzed by electrophoresis on 1.2% agarose gel. The bands were visualized under UV light after staining with ethidium bromide. Each band was measured and analyzed using Gel-Pro Analyzer® software (Media Cybernetics, Inc., Silver Spring, MD).

2.3.5. TNF-α determination

RAW 264.7 cells (1×10^6/mL) were seeded into a series of wells in a 24-well plate and BR, ZY, or GB was separately deposited at doses of 10 μg/mL. The seeded plates were co-cultured at 37 °C under 5% CO_2 for 1, 3, 6, 12, and 24 h. At each time interval, the obtained supernatants were subjected to determination of TNF-α concentrations using an ELISA kit (Biosource Co., Carlsbad, CA) according to the protocols provided by the manufacturer.

2.4. Statistics

Determinations of NO and cytokine productions were conducted using the GLM model procedure (SAS Institute, 1996) for statistical analysis. Significance differences among treatments were determined using Duncan's New Multiple Range Test. Values represented in the bar graphs are means ± standard deviation. For *in vitro* results, Student's *t*-test was used for treatment comparisons.

3. Results and discussion

3.1. Molecular weight estimation of β-glucans

When blue dextran and a series of standard pullulans were separately subjected to GPC analysis, a reversely linear relationship ($R^2 = 0.9952$) among log molecular weights of the standards and their corresponding retention times was achieved (Fig. 1A). As analyzed and estimated by the developed GPC protocol, the molecular weight of isolated BR was 1.82×10^6 Da (Fig. 1B1). This was close to that BR is a homopolysaccharide of glucose with a molecular weight 4.875×10^6 Da reported by Selbmann et al. (2003). In that report, a low angle laser light-scattering detector was equipped for analysis and an evaporative light-scattering detector (ELSD) was used in this study. When GB and ZY were respectively subjected to GPC analysis and estimated according to the linear equation, their molecular weights were 5.25×10^5 and 6.25×10^6 Da (Fig. 1B2 and B3). During GPC analysis of the β-glucans, it is of interest to notice that a peak with 11.6 min of retention time was always detected in each of samples (Fig. 1B). As glucose, sucrose and BR solution after dialysis overnight were run concurrently under the same GPC condition, their retention times were 11.6 min (data not shown). For the GPC column used in this study, the molecular weight resolution range is from 10^3 to 10^6 Da, the small carbohydrate molecules , such as glucose, sucrose and other small sugars could be sensitively detected but not resolved. In particular, BR as a fungal and exocellular β-glucan with molecular weight larger than 10^6 Da and biosynthesized in 36-h cultivation is of merit in mass production by commercialized scale-up fermentation.

3.2. Toxicological assessment of rats subjected to gavage-administration with BR

When Sprague–Dawley rats were gavage-administered with crude BR, at doses of 0, 1.25, 12.5 and 125 mg/kg BW per day for 28 d, their BW increased steadily and similarly, and their relative organ weights were not affected by BR treatment (data not shown). In general, there was no indication of adverse effects of BR on rats during the 28-d *in vivo* experiment. As shown in Table 1, the average hematological values including white blood cells (WBC; within $6–17 \times 10^3$/μL), red blood cells (RBC; within $7–10 \times 10^6$/mL), hemoglobin (Hb; within 11–18 g/dL), and hematocrit (Hct; within 36–49%) for all treatment groups were within the normal ranges reported by Sharp (1998). Moreover, there were no abnormal values for plasma AST (aspartate aminotransferase), ALT (alanine

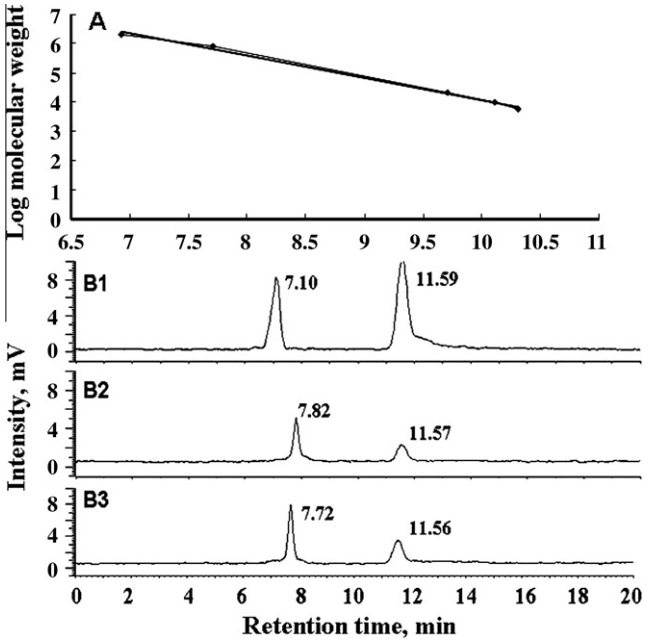


Fig. 1. Molecular weight estimation of β-glucans by gel permeation chromatography (GPC): (A) a linear regress of logarithms of standard polysaccharides and their GPC retention times; (B1) GPC chromatogram of botryosphaeran (BR); (B2) GPC chromatogram of zymosan (ZY); and (B3) GPC chromatogram of barley β-glucan (GB).

Table 1
Changes of hematological parameters and blood biochemical values of Sprague–Dawley rats after subjected to gavage-administration with botryosphaeran with doses of 0, 1.25, 12.5 and 125 mg/kg BW for 28 d.

Items	Normal range	Treatment group[B]			
		Control	1.25 mg/kg	12.5 mg/kg	125 mg/kg
Hematological analysis					
WBC[A] ($10^3/\mu L$)	6–17	10.20 ± 2.47	11.49 ± 2.41	10.54 ± 1.99	11.3 ± 3.15
RBC ($10^6/\mu L$)	7–10	8.95 ± 0.66[a]	8.92 ± 0.73[a]	8.23 ± 0.43[b]	8.93 ± 0.44[a]
HB (g/dL)	11–18	16.56 ± 0.64[a]	16.82 ± 1.32[a]	15.51 ± 0.97[b]	16.64 ± 0.75[a]
HCT (%)	36–49	50.67 ± 2.68[a]	51.70 ± 4.44[a]	47.04 ± 2.80[b]	51.06 ± 2.43[a]
Serum biochemical analysis					
AST (U/L)	110 ± 21	115.70 ± 30.29[ab]	111.40 ± 18.26[ab]	94.60 ± 21.99[b]	118.40 ± 25.72[a]
ALT (U/L)	58 ± 6	48.20 ± 19.29[ab]	48.70 ± 8.57[a]	36.50 ± 5.40[b]	45.10 ± 14.91[ab]
T-P (g/dL)	5.2 ± 0.2	6.74 ± 0.37	6.76 ± 0.29	6.59 ± 0.23	6.79 ± 0.28
BUN (mg/dL)	14 ± 3.0	16.02 ± 1.42	14.94 ± 3.24	14.54 ± 1.50	15.30 ± 2.19
T-Chol (mg/dL)	40–130	70.50 ± 9.26	71.60 ± 13.96	77.50 ± 11.98	74.70 ± 13.47
TG (mg/dL)	26–145	59.20 ± 20.79[ab]	64.80 ± 18.24[a]	49.10 ± 19.18[ab]	42.00 ± 19.21[b]
LDH (U/L)	159–1045	549.20 ± 286.16	473.60 ± 351.23	378.30 ± 259.93	491.90 ± 193.69

[A] WBC: white blood count; RBC: red blood cell; HB: hemoglobin; HCT: hematocrit; AST: aspartate aminotransferase; ALT: alanine aminotransferase; T-P: total protein; BUN: blood urea nitrogen; T-Chol: total cholesterol; TG: triglyceride; LDH: lactate dehydrogenase.
[B] Data are expressed as the mean ± SD (n = 10). Data with different letters in the same row indicates significant difference between control and treatment groups at $P < 0.05$.

aminotransferase), T-P (total protein), BUN (blood urea nitrogen), T-Chol (total cholesterol), TG (triglyceride), and other serum biochemical analyses following crude BR administration (Table 1). Based on the results of the hematological and serum biochemical analyses, no adverse or toxic effect on blood cells and no serum biochemical-related health hazards were observed in rats after daily gavage-administration with crude BR of up to 125 mg/kg BW for 28 d. When animal were euthanized at the end of study, histopathological examinations of the organs' gross appearances and of H&E-stained organ tissues sections were performed according to the Toxicological Principles for the Safety Assessment of Food Ingredients, 2000. There was no sign of toxicity of oral administration with crude BR up to 125 mg/kg BW per day for 28 d (data supplemented).

3.3. Lymphoblastogenesis as affected by BR administration

In rats orally administered with low and middle level (1.25 mg–12.5 mg/kg BW) crude BR with added Con A, LPS, and PMA/ION stimulators for 28 d, lymphoblastogenesis significantly increased ($P < 0.05$) (Fig. 2). However, the increase of rats orally administered with these stimulators in the high level (125 mg/kg) BR treatment was insignificant. It has been reported that β-glucan stimulates lymphoblastogenesis possibly via co-stimulatory signals from phagocytic cells and antigen presenting cells (Grunebach et al., 2002). Our results suggest that administration of an appropriate dose of immunomodulators is critical to achieve an optimal immunity. Whether there is an immunosuppressive effect at a high level of BR treatment needs further investigation. In this study, the high dose BR (125 mg/kg BW) was primarily designed to assess toxicological impacts. Based on our results, the optimal oral administration dose of BR to boost cell-mediated immunity would be between 1.25 and 12.5 mg/kg BW. This supports the observations of Miranda et al., (2008) who reported that anticlastogenic activity in mice was enhanced after botryosphaeran administration increased from 7.5 to 30 mg/kg BW.

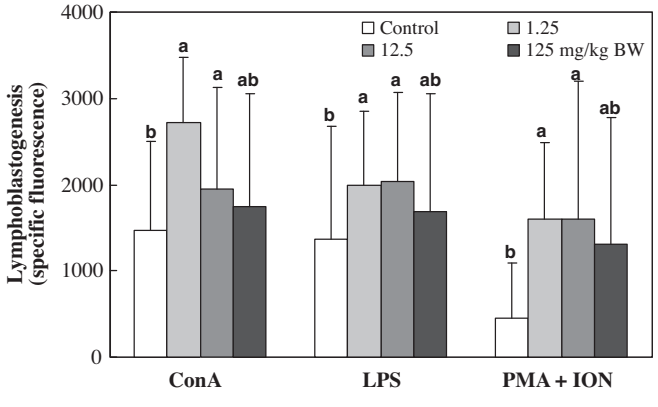
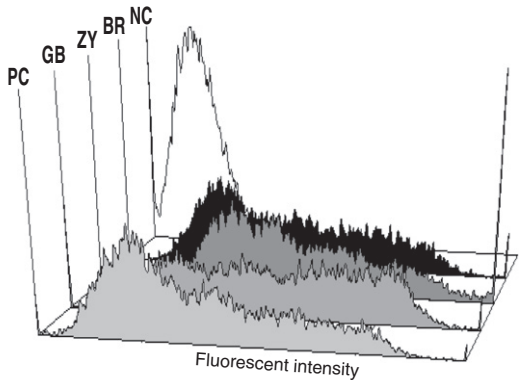


Fig. 2. Lymphoblastogenesis activities, as stimulated by ConA, LPS, and PMA/ION, of lymphocytes of Sprague–Dawley rats after gavage-administration with botryosphaeran at dose levels of 0, 1.25, 12.5 and 125 mg/kg BW per day for 28 d. Data are expressed as means ± SD (n = 6 per treatment). Different letters indicate significant differences between dosages within a group. The level of significance between controls and dosages is $P < 0.05$.

3.4. Phagocytic activity of RAW 264.7 macrophages as affected by BR

For further *in vitro* assessment of immunomodulatory activities by RAW 264.7 macrophages, laboratory purified BR and commer-



NC –macrophages (RAW 264.7 cells) only negative control
BR – macrophages treated with β-glucan from *B. rhodina* + *E. coli*[*]
ZY – macrophages treated with β-glucan from yeast + *E. coli*[*]
GB – macrophages treated with β-glucan from barley + *E. coli*[*]
PC – macrophages + *E. coli*[*]

Fig. 3. Phagocytosis activities on fluorescence-labeled *E. coli* cells by RAW 264.7 macrophage cells as affected by introduction of botryosphaeran (BR), zymosan (ZY) or barley β-glucan (GB). The RAW 264.7 macrophage cells were cultured alone (negative control, NC) or with fluorescence-labeled *E. coli* (positive control, PC). Data are presented as flow cytometric histograms.

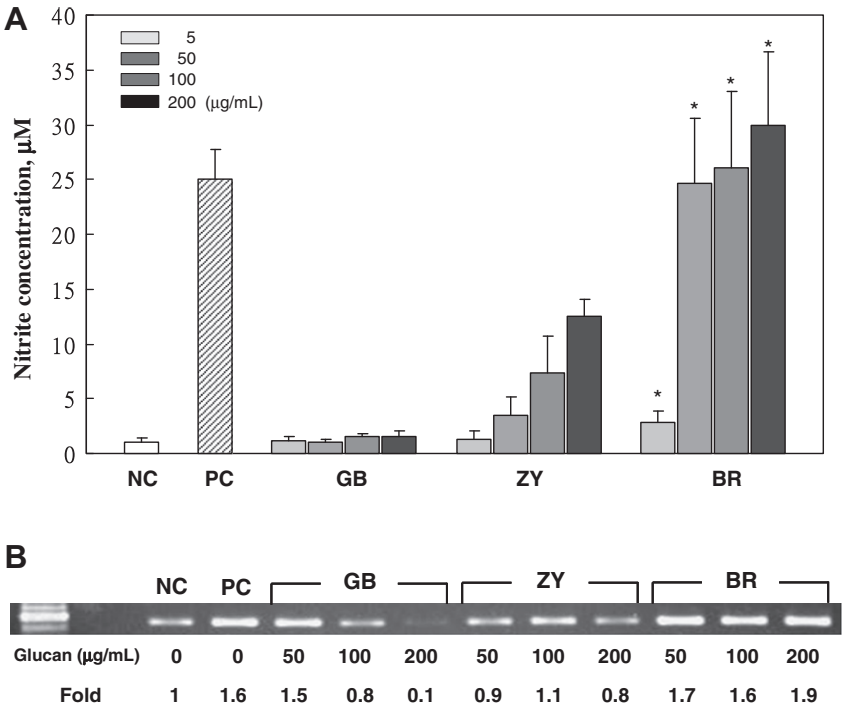


Fig. 4. Nitric oxide (NO) production by RAW 264.7 macrophage cells as affected by introduction of different levels of botryosphaeran (BR), zymosan (ZY) or barley β-glucan (GB) (4A). Macrophages incubated in medium without any β-glucan treatment were negative controls (NC). Macrophages stimulated with LPS (50 ng/mL) were positive controls (PC). Column data are means ± SD from triplicate measurements from three independent assays. Significant differences between the negative control and the treatment groups are expressed as $*$, $P < 0.05$ and $**$, $P < 0.001$. Similarly, expression of *iNOS* mRNA of RAW cells as affected by different β-glucans were demonstrated (4B). The resulting bands were semi-quantified based on density and fold increases indicates the ratios of treatment results to that of the control group.

cial ZY and GB were used in a comparative investigation. All three β-glucans were found to be effective in stimulating phagocytosis of RAW 264.7 cells against fluorescence-stained *E. coli* cells (Fig. 3). GB, the botanical-source β-glucan treated macrophages had slightly higher phagocytosis activity than those of BR and ZY, β-glucans from microbial sources. Stimulation of macrophage phagocytosis and activation of cytokine production through the dectin-1 mediated signaling pathway by ZY and GB have been previously demonstrated (Brown et al., 2003; Tada et al., 2009). Our results support those observations, and it is apparent that the phagocytotic activities of RAW 264.7 cells can be enhanced by pre-incubation with BR, ZY, or GB.

3.5. NO production of RAW 264.7 macrophages as affected by BR

NO is a primary reactive oxygen metabolite exhibiting oxygen-dependent bactericidal activity and it also acts as a signaling mediator for cytokines productions (Mocellin et al., 2007). Here, when RAW 264.7 cells were separately incubated with ZY, GB, or BR at concentrations of 5, 50, 100 or 200 μg/mL, the detected extracellular NO contents in the supernatants collected at 48 h varied markedly (Fig. 4A). The figure indicates that ZY and BR were more potent than GB in stimulation of NO production. Moreover, BR-treated RAW 264.7 macrophages produced more NO than did ZY-treated cells. For example, at the 50 and 100 μg/mL levels, NO concentrations from the BR-treated cells were over three-folds higher than those produced by the ZY-treated cells. This is in agreement with another fungal β-glucan study that showed enhanced NO production following β-glucan treatment (Chen and Seviour, 2007). Here, however, NO production by the GB-treated RAW 264.7 macrophage cells was either at a trace level or was not-detectable. This suggests that NO production may be dependent on β-glucan origin and/or its structure.

Differences in the synthesis of NO production related to the three β-glucans in this study was assessed by quantification of *iNOS* mRNA (Fig. 4B). A dose-dependent suppression by GB treatment on *iNOS* mRNA expression was also observed. In comparison to the negative control, there were no significant changes in efficacy on *iNOS* mRNA expression resulting from the three tested ZY doses. However, for BR doses from 50 to 200 μg/mL, all induced similar increased levels of *iNOS* mRNA expression. The fold increases in BR (1.6–1.9) were similar to that of positive control (1.6) and were markedly higher than that of the negative control (1.0). The results showing higher *iNOS* mRNA expression using BR supports our results showing higher NO production using BR treatment.

3.6. Activation of TNF-α production by BR

The TNF-α concentrations secreted by RAW 264.7 macrophage cells as affected by BR, ZY, or GB treatments at 10 μg/mL for 24 h are shown in Fig. 5. TNF-α production by the ZY-treated cells reached a maximum at 12 h while BR-treated maximum occurred at 3 h. Both BR and ZY at concentrations of 10 μg/mL were capable of stimulating TNF-α production of more than 500 pg/mL within 6 h. Compared to the negative control, GB treatment did not result in additional TNF-α production. Again, the stimulation activity of GB, a β-glucan of cereal origin, was different from that of BR and ZY, both of which are fungal β-glucans. We speculate that this difference is linked to their origins and the nature of their structure–bioactivity characteristics. ZY has been suggested as an adjuvant in DNA vaccines related to the control of HIV and for use in conjunction with other anti-cancer therapies (Ara et al., 2001; Li et al., 2007). ZY is also widely used in immunocompromised patients during radio- and chemo-therapy (Hunter et al., 2002). Based on its microbial origin, and the results obtained in this study, the

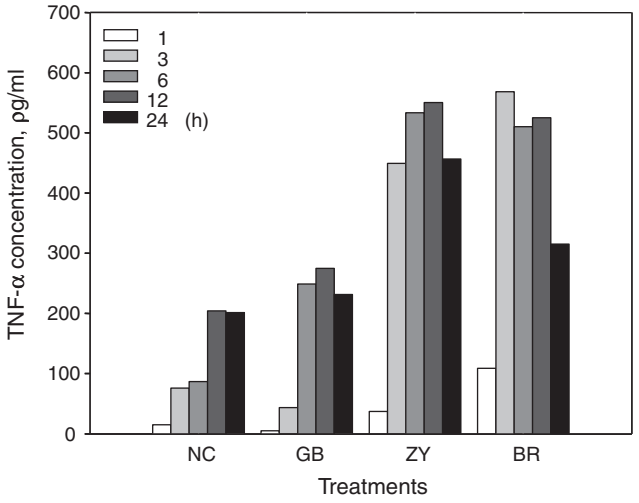


Fig. 5. Productions of TNF-α as affected by introduction of 10 μg/mL botryosphaeran (BR), zymosan (ZY) or barley β-glucan (GB) to RAW 264.7 macrophage cells were measured over a 24 h period at 1, 3, 6, 12, and 24 h. There were dramatic increases in TNF-α productions after 3 h in BR and ZY treated macrophages, where as GB had a similar profile as that of control group.

active motif in BR might be structurally similar to that in the ZY, also from a microbial source. Thus, BR may feature similar immunomodulatory properties to those of ZY.

4. Conclusion

Botryosphaeran (BR) is a newly emerged exocellular β-glucan with 1.82×10^6 Da of molecular weight estimated by GPC analysis. Based on its reported composition and elucidated structure, it seems unlikely to regard BR as a toxic ingredient to be ingested. With an attempt to provide science-based assessments of its safety and based on the results of our *in vivo* experiments, we have further shown that BR to be toxicologically acceptable. Nevertheless, for further toxicological assessments of BR, conductions of a subchronic toxicity study (90 d) and/or on non-rodent species are needed prior to subjection of BR for human consumption. Based on our immunomodulation results from *in vivo* experiments with rats fed BR and *in vitro* studies with RAW 264.7 cells treated with BR, it is apparent that BR-treatment possesses potent immunomodulatory properties that can boost lymphoblastogenesis. *In vivo* activation might be associated with enhancement of NO production, *iNOS* expression, and TNF-α production as was observed in the *in vitro* experiments with RAW 264.7 macrophage cells. In comparison with other β-glucans, BR and ZY, both fungal glucans, produced similar immunomodulation results. Moreover, the two fungal glucans produced results distinct from those of GB, a botanical origin β-glucan. The observed variation in bioactivities appears to be dependent on the origin of the glucan and/or the nature of its structure–activity characteristics. Based on the results showing that BR possesses immunomodulatory properties as potent as ZY, and supported by the *in vitro* and *in vivo* evidence of its toxicologically acceptable nature, BR is a of potency candidate recommended for further development of BR-containing nutraceuticals and health-benefit related products.

Conflict of Interest

No conflict of interest was declared.

Acknowledgements

The financial support of the National Science Council (NSC 95-2313-B415-003 and NSC 97-2321-B415-001), Republic of China, the helpful assistance in the laboratory of J.-C. Chang, J.-T. Chen and G.-T. Liu, and the professional English editing by Wallace Institute, Hsingchu, Taiwan, are acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fct.2010.10.036.

References

Ara, Y., Saito, T., Takagi, T., Hagiwara, E., Miyagi, Y., Sugiyama, M., Kawamoto, S., Ishii, N., Yoshida, T., Hanashi, D., Koshino, T., Okada, H., Okuda, K., 2001. Zymosan enhances the immune response to DNA vaccine for human immunodeficiency virus type-1 through the activation of complement system. Immunology 103, 98–105.

Barbosa, A.M., Steluti, R.M., Dekker, R.F., Cardoso, M.S., Corradi da Silva, M.L., 2003. Structural characterization of botryosphaeran: a (1–>3; 1–>6)-beta-D-glucan produced by the ascomyceteous fungus, *Botryosphaeria* sp. Carbohydr. Res. 338, 1691–1698.

Brown, G.D., Herre, J., Williams, D.L., Willment, J.A., Marshall, A.S.J., Gordon, S., 2003. Dectin-1 mediates the biological effects of β-glucans. J. Exp. Med. 197, 1119–1124.

Chen, J., Seviour, R., 2007. Medicinal importance of fungal β-(1,3)(1,6)-glucans. Mycol. Res. 111, 635–652.

Crognale, S., Bruno, M., Fidaleo, M., Mores, M., Petruccioli, M., 2006. Production of β-glucan and related glucan-hydrolases by *Botryosphaeria rhodina*. J. Appl. Microbiol. 102, 860–871.

Du, Z., Kelly, E., Mecklenbrauker, I., Agle, L., Herrero, C., Paik, P., Ivashkiv, L.B., 2006. Selective regulation of IL-10 signaling and function by zymosan. J. Immunol. 176, 4785–4792.

Goldman, R., Ferber, E., Meller, R., Zor, U., 1994. A role for reactive oxygen species in zymosan and β-glucan induced protein tyrosine phosphorylation and phospholipase A_2 activation in murine macrophages. Biochem. Biophys. Acta 1222, 265–276.

Grunebach, F., Weck, M.M., Reichert, J., Brossart, P., 2002. Molecular and functional characterization of human dectin-1. Exp. Hematol. 30, 1309–1315.

Hunter Jr., K.W., Gault, R.A., Berner, M.D., 2002. Preparation of microparticulate beta-glucan from *Saccharomyces cerevisiae* for use in immune potentiation. Lett. Appl. Microbiol. 35, 267–271.

Kalra, S., Jood, S., 2000. Effect of dietary barley derived beta-glucan on cholesterol and lipoprotein fractions in rats. J. Cereal Sci. 31, 141–145.

Lee, J.Y., Kim, J.Y., Lee, Y.G., Rhee, M.H., Hong, E.K., Cho, J.Y., 2008. Molecular mechanism of macrophage activation by exopolysaccharides from liquid culture of *Lentinus edodes*. J. Microbiol. Biotechnol. 18, 355–364.

Lehmann, A.K., Sornes, S., Halstensen, A., 2000. Phagocytosis: measurement by flow cytometry. J. Immunol. Methods 243, 229–242.

Li, B., Allendorf, D.J., Hansen, R., Marroquin, J., Cramer, D.E., Harris, C.L., 2007. Combined yeast beta-glucan and antitumor monoclonal antibody therapy requires C5a-mediated neutrophil chemotaxis via regulation of decay-accelerating factor CD55. J. Cancer Res. 67, 7421–7430.

Ljungman, A.G., Leanderson, P., Tagesson, C., 1998. (1-3)-β-D-Glucan stimulates nitric oxide generation and cytokine mRNA expression in macrophages. Environ. Toxicol. Pharmacol. 5, 273–281.

Lowry, V.K., Farnell, M.B., Ferro, P.J., Swaggerty, C.L., Bahl, A., Kogut, M.H., 2005. Purified β-glucan as an abiotic feed additive up-regulates the innate immune response in immature chickens against *Salmonella enterica* serovar Enteritidis. Int. J. Food Microbiol. 98, 309–318.

Mendes, S.F., Santos Jr., O.D., Barbosa, A.M., Vasconcelos, A.F.D., Aranda-Selverio, G., Monteiro, N.K., Dekker, R.F.H., Pereira, M.S., Tovar, A.M.F., Mourao, P.A.D.S., Corradi da Silva, M.D.L., 2009. Sulfonation and anticoagulant activity of botryosphaeran from *Botryosphaeria rhodina* MAMB-05 grown on fructose. Int. J. Biol. Macromol. 45, 305–309.

Miranda, C.C.B., Dekker, R.F.H., Colus, I., Zaia, C.T., Castro, I., Barbosa, A.M., 2007. Botryosphaeran: a new fungal exopolysaccharide presenting antimutagenic, hypoglycaemic and hypocholesterolaemic activities in mice and rats. J. Biotech. 131S, S58.

Miranda, C.C., Dekker, R.F., Serpeloni, J.M., Fonseca, E.A., Cólus, I.M., Barbosa, A.M., 2008. Anticlastogenic activity exhibited by botryosphaeran, a new exopolysaccharide produced by *Botryosphaeria rhodina* MAMB-05. Int. J. Biol. Macromol. 42, 172–177.

Mocellin, S., Bronte, V., Nitti, D., 2007. Nitric oxide a double edged sword in cancer biology searching for therapeutic opportunities. Med. Res. Rev. 27, 317–352.

Ohno, N., Egawa, Y., Hashimoto, T., Adachi, Y., Yadomae, T., 1996. Effect of beta-glucans on the nitric oxide synthesis by peritoneal macrophage in mice. Biol. Pharm. 19, 608–612.

SAS Institute, 1996. SAS® User's Guide: Statistics. SAS Institute Inc., Cary, NC, USA.

Selbmann, L., Stingele, F., Petruccioli, M., 2003. Exopolysaccharide production by filamentous fungi: the example of *Botryosphaeria rhodina*. Antonie Van Leeuwenhoek 84, 135–145.

Sharp, P.E., 1998. Important biological features. In: Suckow, Mark.A. (Ed.), The Laboratory Rat. CRC Press, New York, pp. 1–15.

Tada, R., Adachi, Y., Ishibashi, K., Tsubaki, K., Ohno, N., 2008. Binding capacity of a barley β-glucan recognition molecule dectin-1. J. Agric. Food Chem. 56, 1442–1450.

Tada, R., Ikeda, F., Aoli, K., Yoshikawa, M., Kato, Y., Adachi, Y., Tanioka, A., Ishibashi, K., Tsubaki, K., Ohno, N., 2009. Barley-derived β-glucan induces immunostimulation via a dectin-1-mediated pathway. Immunol. Lett. 123, 144–148.

Toxicological Principles for the Safety Assessment of Food Ingredients. Chapter IV.C.3.a. Short-term toxicity studies with rodents In: Redbook 2000, Food and Drug Administration, College Park, MD, USA.

Volman, J.J., Ramakers, J.D., Plat, J., 2008. Dietary modulation of immune function by β-glucans. Physiol. Behavior 94, 276–284.

Wu, M.B., Wu, Y.L., Zhou, J., Pan, Y.J., 2009. Structural characterization of a water-soluble polysaccharide with high branches from the leaves of *Taxus chinensis* var. mairei. Food Chem. 113, 1020–1024.