Scientific Research Publishing

Food and Nutrition Sciences, 2023, 14, 341-368
https://www.scirp.org/journal/fns
ISSN Online: 2157-9458
ISSN Print: 2157-944X

Protection of Allergic Asthma in Mice by Black Rice Bran Bioprocessed with Shiitake Mushroom Mycelia

Ki Sun Kwon[1], Woon Sang Hwang[1], Kyung Hee Lee[1], Kyung Jin Kim[1], Wha Young Lee[1], Jeanman Kim[1], Sang Jong Lee[1], Sung Phil Kim[1*], Mendel Friedman[2*]

[1]STR Biotech Co., Ltd., Chuncheon, Republic of Korea
[2]U.S. Department of Agriculture, Western Regional Research Center, Agricultural Research Services, Albany, CA, USA
Email: *spkim@strbiotech.co.kr, *mendel.friedman@usda.gov

How to cite this paper: Kwon, K.S., Hwang, W.S., Lee, K.H., Kim, K.J., Lee, W.Y., Kim, J., Lee, S.J., Kim, S.P. and Friedman, M. (2023) Protection of Allergic Asthma in Mice by Black Rice Bran Bioprocessed with Shiitake Mushroom Mycelia. *Food and Nutrition Sciences*, **14**, 341-368.
https://doi.org/10.4236/fns.2023.144023

Received: March 9, 2023
Accepted: April 23, 2023
Published: April 26, 2023

Abstract

We evaluated the potential of orally fed new food formulations to inhibit biomarkers reported to be involved in the causes of allergic asthma in mice. Asthma, a serious non-communicable disease, affects both adults and children and can be undertreated. New functional foods could provide therapeutic approaches. Here, the anti-asthma mechanism of a new functional food and three isolated fractions produced by bioprocessing black rice bran with shiitake mushroom mycelia was evaluated in mast cells, B cells, and orally fed mice and compared with non-bioprocessed black rice bran. *In vitro*, the treatments inhibited RBL-2H3 cell degranulation and immunoglobulin E (IgE) production. The *in vitro* anti-asthma effects were confirmed in orally fed mice following asthma induction by alumina and chicken egg ovalbumin (OVA). The suppression of asthma resulted from the inhibition of inflammation- and immune-related substances, including OVA-specific IgE, thymic stromal lymphopoietin, eotaxin, leukotriene C4, prostaglandin D2, and vascular cell adhesion molecule-1 in bronchoalveolar lavage fluid and serum. The treatment also reversed the thickening of the lung airway wall. The inflammation and asthma inhibition seems to be regulated by the balance of the T-helper cells' Th1/Th2 immune response and the inhibition of multiple biomarkers associated with the cause of asthma. Future human clinical studies with adults and children should determine the potential therapeutic value of the anti-asthma effects of the new functional foods.

Keywords

Black Rice Bran, Shiitake Mushrooms, Bioprocessed Functional Food, Mast Cells, Mice Feeding Studies, Bioassays, Cytokines, Immunoglobulins,

Bronchial Lavage Fluid, Histology, Anti-Inflammatory Effect, Asthma Prevention, Research Needs

1. Introduction

Asthma is a lung disease characterized by chronic inflammation of airway-hypersensitivity mucus overproduction, airway remodeling, and airway narrowing that affects more than 300 million people worldwide and an estimated 7 million children in the United States. Allergic asthma, also known as severe asthma (SA), is an inflammatory incurable disease of the lungs and associated airways characterized by the infiltration of immune cells that is difficult to treat and manage [1] [2] [3]. Multiple factors, including genetics, air pollution, smoking, obesity, and consumption of ultra-processed food, are reported to contribute to the cause (etiology) of the disease. The prevalence, severity, and etiology of asthma have stimulated studies on the identification of genetic and molecular biomarkers that may be associated with immune infiltration and signaling pathways. To contribute to this effort, Jiang *et al.* (2022) used bioinformatic methods to identify genes and signaling pathways that were affected by the differences in immune cell infiltration that is characteristic of asthma [4]. They identified key genes and immune cell infiltration patterns involved in asthma progression, suggesting that the results may provide a better understanding of the relationship between airway epithelial transcriptome and clinical data. In a related study, Zhang *et al.* (2022) reported that specific signaling pathways and autophagy-related differentially expressed genes (DEGs) are potential diagnostic biomarkers in the cause and progression of asthma [5]. The authors suggest that their results expand our understanding of asthma and that the inhibition of the gene ERN1 might be a useful target in the treatment of asthma. In a survey of pre-inflammatory asthma biomarkers (IgE, IL-4, and IL-13), Zahedi *et al.* (2022) found that children residing in industrial areas are prone to asthma allergy, miRNA mutation, and other chronic diseases [6].

As part of an effort to discover new anti-asthma functional foods, we previously explored in two published studies on the mechanism of allergic asthma formation and inhibition in cells and in mice. In the first study, we describe the mechanism of protection against allergic asthma by bioactive elm tree *Ulmus parvifolia* bark bioprocessed (fermented) with shiitake mushroom mycelia in culture [7]. The results showed that oral administration of the new functional food ameliorated biomarkers associated with allergic asthma. These included the reduction of elevated T-helper cell Th1 cytokine production to near normal levels, reduced expression of inflammatory mediators, reduced immunoglobulin production related to the Th1 and Th2 immune reaction, reduced elevated Treg IL-10 cytokine formation to near normal levels, reduced number of lymphocytes in bronchoalveolar lavage fluid (BALF), suppression of lung airway inflammation, and a protective effect on the morphology of lung tissues against inflam-

matory cell infiltration. The second study on the anti-asthma effect of the edible *Ecklonia cava* alga bioprocessed with mushroom mycelia showed similar beneficial molecular, cellular, and histological results [1].

Black rice bran that has been bioprocessed through fermentation with mushroom contains polysaccharides with reported physiological, health-promoting activities including the inhibition of infections by the foodborne pathogen *Salmonella* Typhimurium in mice [8] and the inhibition of lipopolysaccharide (LPS)-induced endotoxemia in mice [9] via the activation of the Th1 immune response *in vivo*. These observations and the fact that black rice bran and mushrooms are reported to contain several biologically active, health-promoting compounds, such as *γ*-oryzanol in the case of black rice bran and flavonoids and mushrooms contain bioactive polysaccharides motivated us to determine the anti-asthma effect of another functional food prepared by bioprocessing black rice (*Oryza sativa* L.) bran with shiitake mushroom (*Lentinulus edodes*) mycelia, as well as fractions isolated by preparative chromatography, the objective of the present study.

2. Materials and Methods

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), chicken egg ovalbumin (OVA, grade V), and aluminum hydroxide were purchased from Merck (Darmstadt, Germany). Phosphate-buffered saline (PBS), fetal bovine serum (FBS), and other cell culture reagents were purchased from Gibco BRL (Grand Island, NY). IL-4 mouse enzyme-linked immunosorbent assay (ELISA) kit, IL-5 mouse ELISA kit, and other ELISA kits were purchased from R&D systems (Minneapolis, MN, USA). *γ*-Oryzanol was obtained from Wako Pure Chemical Corporation (Osaka, Japan), and arabinose, galactose, glucose, mannose, and xylose were obtained from Merck. High-performance liquid chromatography (HPLC) experiments were performed with an instrument consisting of a Shimadzu LC-20 with a photodiode array detector, refractive index detector, and binary pump with Shimadzu shim-pack GIS CN (150 × 4.6 mm, 5 μm) column and Bio-rad Aminex HPX-87P (300 × 7.8 mm, 9 μm, lead form).

2.2. BRB-F Preparation and Purification Processes

The following samples were prepared for the evaluation of anti-asthma properties: BRB-F, bioprocessed (fermented) black rice bran/mushroom mycelia; BRB-F-S, supernatant fraction of BRB-F; BRB-F-W, water-soluble fraction of BRB-F; BRB-F-P, polysaccharide fraction of BRB-F (**Figure 1**). BRB-F was produced according to the previously published method [10], with minor modifications. Lentinus edodes fungal mycelia were cultured on a potato dextrose agar (PDA) medium. The mycelium cultured in PDA media was inoculated into 50 mL of the liquid medium. Incubation experiments were performed in 250 mL Erlenmeyer flasks for 5 days at 28˚C on a rotary shaker (120 rpm) and used to

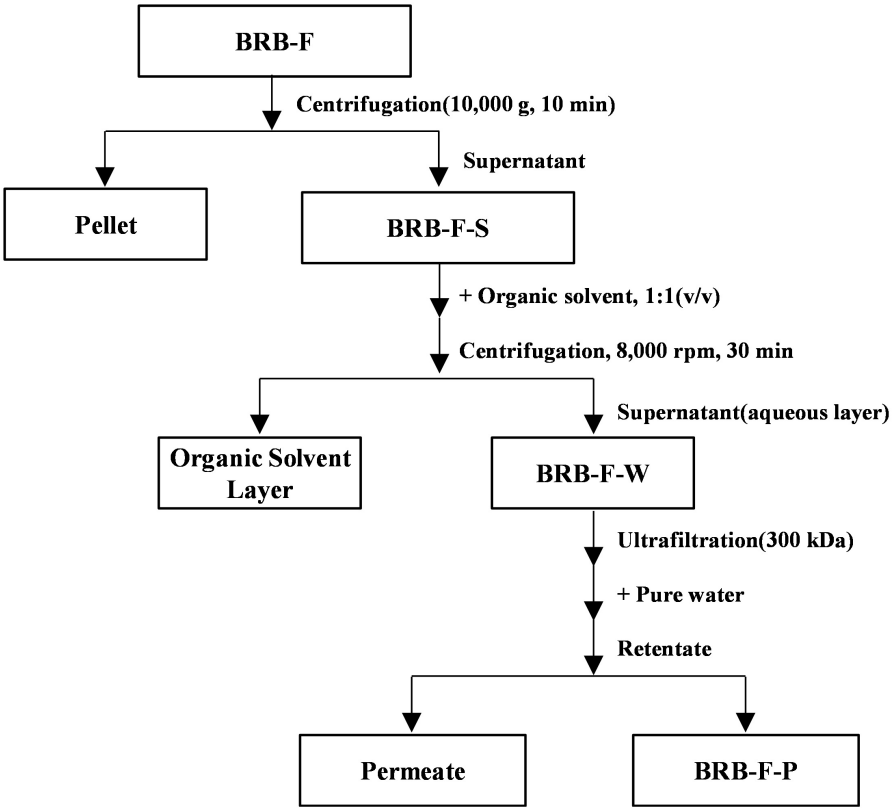

Figure 1. Scheme of the purification procedure of bioprocessed black rice bran.

seed the main liquid culture. The main liquid medium contained black rice bran (100 g/L). Subsequently, the medium was treated with amylase at 60˚C for 60 minutes for enzymatic digestion of particulate matter containing carbohydrates. The culture was then adjusted to pH 6.0 using HCl, and then sterilized in the autoclave. Experiments on the main liquid culture were started by inoculating the inoculum (10%) of pre-cultured mycelium using a 5 L fermenter (working volume of 3 L) at 28˚C and 150 rpm. After 3 days, the culture mass was treated with amylase and an enzyme mixture for cell wall lysis containing cellulose, hemi-cellulase, pectinase, glucanase, mannose, and arabinase at 50˚C for 60 min. Subsequently, enzyme-treated cultures were extracted with hot water at 90˚C for 1 h and lyophilized with a solid material. BRB-F-S was obtained by removing insoluble residues from BRB-F, and BRB-F-W was further purified by removing formed submicro-size lipid particles. BRB-F-P was sequentially purified by removing small-molecular impurities from BRB-F-W. Briefly explaining each step, the bioprocessed black rice bran (BRB-F) was centrifuged (10,000 g, 10 min, 4˚C) and the supernatant was recovered to obtain BRB-F-S. Thereafter, nano-size lipid particle impurities contained in BRB-F-S were extracted with methylene chloride, and an aqueous solution was obtained through liquid-liquid extraction to purify BRB-F-W. Low-molecular-weight impurities contained in BRB-F-W were removed by ultrafiltration (300 kDa cut-off), and a high-molecular fraction (UF retentate) was recovered to obtain BRB-F-P (Figure 1).

2.3. Analysis of γ-Oryzanol in the Purification Steps of BRB-F

A 0.5 g sample of each preparation was added to 50 mL of ethanol. Ultrasonic extraction was then performed for 1 hour. The supernatant was collected and analyzed by filtering with a 0.45 μm syringe filter. Table 1 shows the analysis conditions for the standard compound using HPLC.

2.4. Analysis of Component Sugars of BRB-F-P

A 40 mg sample of BRB-F-P was dissolved in 10 mL of 4% sulfuric acid solution and hydrolyzed at 120˚C for 2 hours. Calcium carbonate was added to the hydrolyzed sample to adjust the pH to 5 - 6. The supernatant of the pH-adjusted sample was obtained and analyzed by filtering with a 0.45 μm syringe filter. The analysis conditions of component sugars using HPLC are shown in Table 2.

2.5. RBL-2H3 Cell Culture and β-Hexosaminidase Release Assay

The transformed rat basophilic leukemia mast cell line RBL-2H3 from the American Type Tissue Culture Collection (ATCC, Manassas, VA, USA) was cultured in a modified DMEM containing 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, and 10% heat-inactivated FBS. Penicillin (100 U/mL) and streptomycin (100 mg/mL) were also added to the medium. Cells were cultured at 37˚C in a humidified atmosphere with 5% CO_2. As an indicator of an allergic reaction, β-hexosaminidase secretion in the RBL-2H3 cell line was determined using a published method [11]. Briefly,

Table 1. Analysis method for γ-oryzanol by HPLC-PDA.

Instrument		Shimadzu LC system		
Sampler temp.		10˚C		
Injection volume		20 μL		
Rinse solution		50% MeOH		
Mobile phase	Mobile Phase A	IPA		
	Mobile Phase B	Hexane (1% acetic acid)		
		Time (min)	A (%)	B (%)
		0	5	95
		10	5	95
	Gradient	11	100	0
		15	100	0
		16	5	95
		30	5	95
Flow rate		0.5 mL/min		
Column		Shimadzu Shim-pack GIS CN 150 × 4.6 mm 5 μm		
Column oven temp.		30˚C		
Detector		Photodiode array (PDA), 320 nm		

Table 2. Analysis method for component sugar by HPLC-RI.

Instrument		Shimadzu LC system		
Sampler temp.		10˚C		
Injection volume		20 μL		
Rinse solution		Water		
Mobile phase	Mobile Phase A	Water		
	Mobile Phase B	–		
	Isocratic	Time (min)	A (%)	B (%)
		0	100	0
		60	100	0
Flow rate		0.3 mL/min		
Column		Aminex HPX-87P 300*7.8 mm		
Column oven temp.		85˚C		
Detector		Refractive index (RI), 55˚C		

RBL-2H3 cells were cultured in a 96-well plate (1×10^5 cells/mL). The samples in tyrode buffer (200 μL; 137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.1 mM $MgCl_2$, 11.9 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 5.6 mM glucose, pH 7.2) were added to each well and incubated for 15 min. The units of concentration are shown in the figure. To stimulate cells after removal of the extracts by washing with tyrode buffer, calcium ionophore A23187 (10 μM) in tyrode buffer was added for 20 min. The supernatant (50 μL) containing released β-hexosaminidase was recovered and it was then mixed with the same volume of p-nitrophenyl-N-acetyl-β-glucosaminide solution (1 mM, pH 5.2) and incubated for 1 h at room temperature. The reaction was terminated by adding sodium carbonate buffer (67 mM, pH 10.2). The absorbance of the supernatant was read at 405 nm using a microplate reader (VersaMax, Molecular Devices Corp., CA, USA).

2.6. U266.B1 Cell Culture and IgE Production

The U266.B1 human multiple myeloma B lymphocyte cells from the American Type Tissue Culture Collection (ATCC, Manassas, VA, USA) were cultured in a modified RPMI1640 medium containing 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, and 15% heat-inactivated FBS. Penicillin (100 U/mL) and streptomycin (100 mg/mL) were also added to the medium. Cells were cultured at 37˚C in a humidified atmosphere with 5% CO_2. To assess changes in IgE production levels, U266.B1 cells were stimulated with 10 μg/mL lipopolysaccharide (LPS), 5 ng/mL human IL-4, and either of the samples for 72 h. The culture supernatants were recovered for IgE assay using a commercial kit (Biorbyt, San Francisco, CA, USA) according to the manufacturer's instructions. Absorbance of the final reaction mixture was read at 450 nm using a microplate reader.

2.7. BEAS-2B Cell Culture and Cell Stimulation

The BEAS-2B human bronchial epithelial cells from the American Type Tissue Culture Collection (ATCC, Manassas, VA, USA) were cultured in a modified DMEM containing 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, and 10% heat-inactivated FBS. Penicillin (100 U/mL) and streptomycin (100 mg/mL) were also added to the medium. Cells were cultured at 37˚C in a humidified atmosphere with 5% CO_2. To measure chemokine production, BEAS-2B cells were cultured in a 6-well plate (5×10^5 cells/well). Once cells had reached confluence, the cells were pretreated with 1 mL serum-free medium containing BRB-F-S (10 μg/mL) for 48 h. Then, 10 ng/mL tumor necrosis factor (TNF)-*α* was treated for 24 h to produce RANTES. Other cells were incubated for 48 h with 50 ng/mL interleukin (IL)-4 + TNF-*α* to produce eotaxin and eotaxin-3. Cell supernatants were collected after 24 or 48 h for ELISA.

2.8. Animals

Pathogen-free female Balb/c mice, 6 weeks old, were purchased from Koatech (Gyunggi-do, Korea). The mice were housed in a stainless-steel cage under a 12 h light/dark cycle with a temperature range of 23˚C ± 3˚C and relative humidity of 50% ± 10%. Mice were fed the pelletized normal commercial chow diet (Cat. No. 5L79, Orient Bio, USA) and tap water *ad libitum* for 1 week after arrival for acclimation.

2.9. Antigen Sensitization, Challenge, and Treatment

The protocol for sensitization and inhalational challenge was performed according to the method of Temelkovski *et al.* [12] (1998) with slight modifications. Briefly, acclimatized Balb/c mice were arbitrarily divided into the following groups (n = 10), avoiding any intergroup difference in body weight: Normal mice, OVA only, BRB, BRB-F and its purified fractions-treated groups (BRB—extracts of BRB; BRB-F—bioprocessed product; and BRB-F-S, BRB-F-W and BRB-F-P—isolated fractions of bioprocessed product). Mice in the OVA sensitization/challenge group were intraperitoneally sensitized with 20 μg of OVA emulsified with 0.2 mL of 1 mg aluminum hydroxide in phosphate-buffered saline (PBS, pH 7.4). Mice in the vehicle group were intraperitoneally sensitized with phosphate-buffered saline (PBS, 0.2 mL, pH 7.4). Injections were performed three times on days 1, 8, and 15. The sensitized mice were subjected to OVA challenge by placing each mouse individually in a Plexiglass box (43.5 × 27.5 × 31.5 cm). Challenge was continued with repeated exposure to an aerosol of 1% OVA using an ultrasonic nebulizer (NE-U12, Omron Co., Kyoto, Japan). Mice were then similarly exposed to OVA for 30 min once a day for 5 consecutive days (day 25 to 29). The vehicle-treated group was subjected to PBS exposure. For the treated groups, mice sensitized/challenged with OVA as described were orally administered with either of the extracts for 14 consecutive days (day

16 to 29). PBS was used as vehicle, and all extracts were administered in the diet. All mice were sacrificed by CO_2 inhalation 24 h after the last (day 30) to assess the asthma inhibitory effect of BRB-F and its purified products. The described experimental design is schematically shown in Figure 2. All experimental procedures were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee at Chuncheon Bioindustry Foundation (CBF IACUC No. 2021-010).

2.10. Collection of Bronchoalveolar Fluid (BALF) and Blood

For the collection of BALF, tracheotomy was performed as follows. The tracheas exposed by cannulating upper tracheas and BALF were carefully collected by twice lavaging with ice-cold PBS (2 mL). Collected lavage fluids were then centrifuged at 2000 g for 1 min at 4˚C. The recovered supernatants were set aside and kept at −70˚C until further analysis. Cell pellets were resuspended in warm RPMI 1640 media, followed by slide preparation by centrifuge (Micro 17R, Hanil Science, Incheon, Republic of Korea) and staining with Wright-Giemsa stain. The slides were observed at ×40 magnification for differential cell counts by counting a total of 300 cells per slide under light microscopy (CKX41, Olympus, Tokyo, Japan). The total cell number in BALF was also measured by microscopic cell counting using a hemocytometer. Blood was collected by cardiac puncture from the sacrificed mice.

2.11. Histological Analysis

The exsanguinated left lung was removed from the chest cavity and fixed with 4% paraformaldehyde in phosphate buffer (0.5 M, pH 7.4). Lobes were isolated, dehydrated with ethanol, and embedded in paraffin. The tissues were then cut to a thickness of 4 μm and mounted onto glass slides. To observe inflammatory cell infiltration, the sections were dewaxed using xylene and ethanol, stained with hematoxylin and eosin Y (H&E), and examined by light microscopy (CKX41, Olympus, Tokyo, Japan).

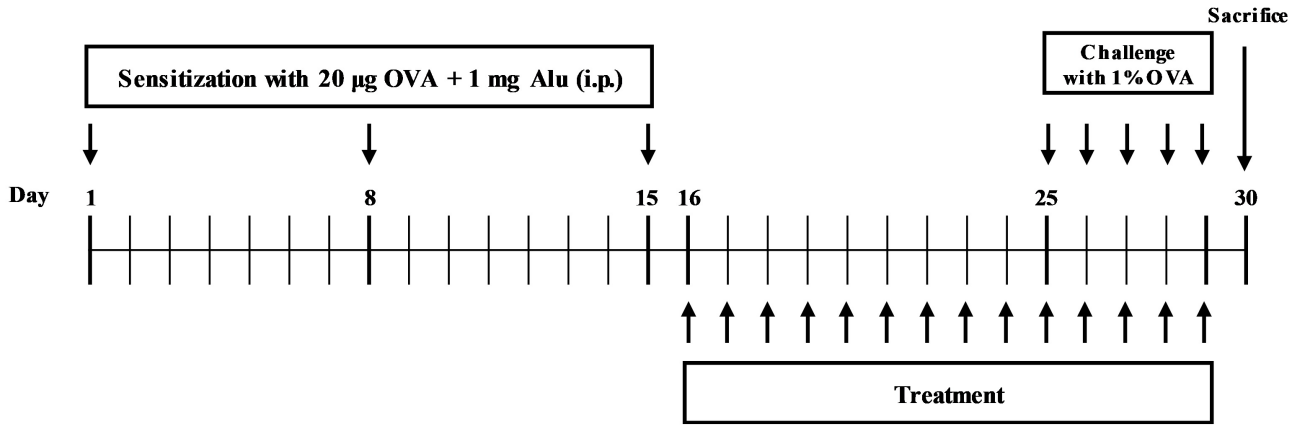

Figure 2. Scheme of OVA-induced asthma mouse model.

2.12. Statistical Analysis

Results are expressed as the mean ± SD of three independent experiments. Significant differences between means were determined by the analysis of variance (ANOVA) test using the Statistical Analysis Software package SAS (Cary, NC, USA). $p < 0.05$ is regarded as significant.

3. Results

3.1. Analysis of γ-Oryzanol in the Purification Steps of BRB-F and Analysis of Component Sugars of BRB-F-P

Because γ-oryzanol is a bioactive compound in black rice bran, we determined its content in BRB-F and its isolated fractions BRB-F-S, and BRB-F-W. γ-Oryzanol was present in BRB-F, but its content decreased through the purification steps. It was not detected in the polysaccharide fraction, BRB-F-P (Figure 3 and Table 3). The content of component sugars in the polysaccharide fraction BRB-F-P was 96%, with the main polysaccharide constituents being glucose and galactose (Figure 4 and Table 4).

3.2. Inhibitory Effect of BRB-F and Its Isolated Fractions on Mast Cell Activation

β-hexosaminidase is stored in the granules of mast cells and is degranulated and secreted when mast cells generate immune activity. To confirm the anti-allergic effect, the inhibition of β-hexosaminidase secretion, which is an indicator of degranulation of cells during an allergic reaction, was observed using the RBL-2H3 mast cell line as the main allergic response cell. In RBL-2H3 cells, degranulation was induced by treatment with A23187 and the amount of β-hexosaminidase was increased. Upon treatment with either 100 μg/mL of BRB or BRB-F, a 50% inhibitory effect was confirmed. The inhibitory activity was increased in a dose-dependent. The units of concentration are shown in Figure 5 and Figure 6.

Table 3. Analysis of γ-oryzanol in BRB-F and its isolated fractions.

Sample	Retention time (min)	Area	Cal conc. (mg/L)	Cal cont. (mg/g)
BRB-F	5.987	2286906	42.375	4.11
BRB-F-S	5.973	1046065	19.081	1.85
BRB-F-W	5.972	119891	<LLOQ	<LLOQ
BRB-F-P	0.000	0	0.000	0.00

Table 4. Analysis of component sugars in BRB-F-P.

Sample	Glucose	Xylose	Galactose	Arabinose	Mannose	Total
	Cal cont. (mg/g)	Cal cont. (mg/g)	Cal cont. (mg/g)	Cal cont. (mg/g)	Cal cont. (mg/g)	Cal cont. (mg/g)
BRB-F-P	885.50	<LLOQ	77.00	<LLOQ	0.00	962.50

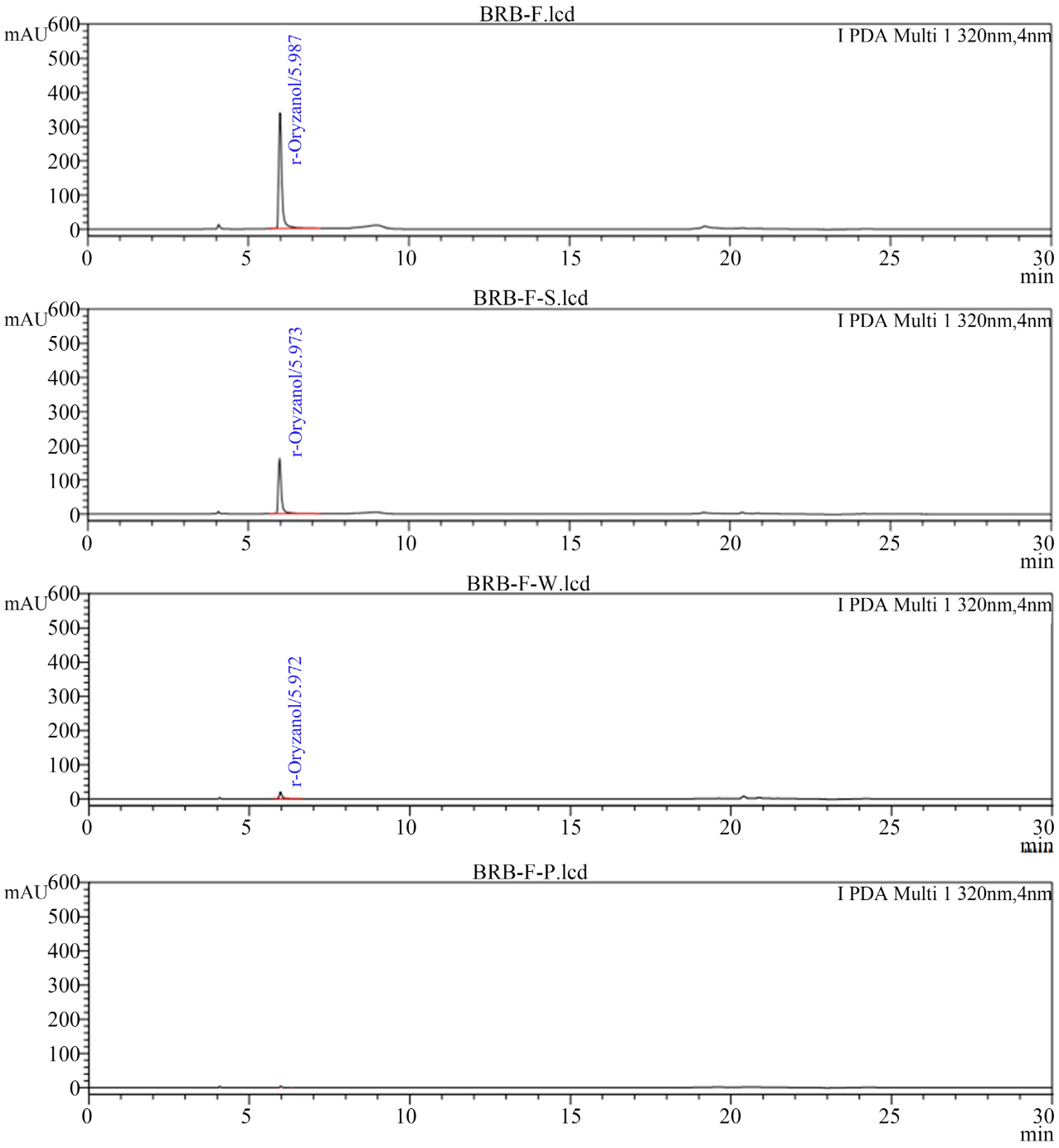

Figure 3. Analysis chromatogram of γ-oryzanol in BRB-F and its isolated fractions.

However, the β-hexosaminidase secretion inhibitory activity gradually decreased according to the sequential purification process of BRB-F, and thus no activity was observed in the final BRB-F-P purification fraction (**Figure 5**).

3.3. Inhibition of IgE Production in a B Cell Line by BRB-F and Its Isolated Fractions

The inhibitory effect of the bioprocessed products on the levels of IgE secreted

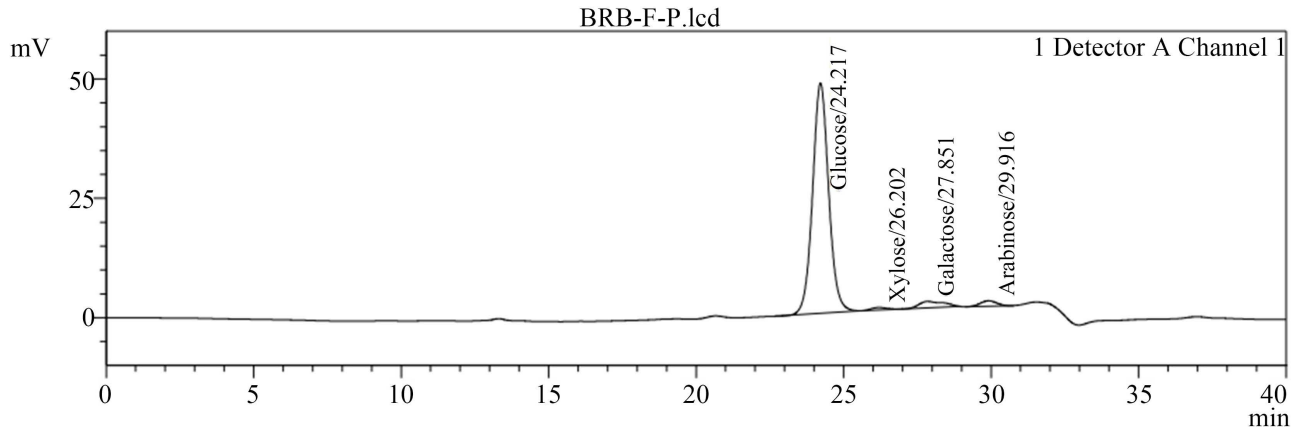

Figure 4. Analysis chromatogram of component sugars in BRB-F-P.

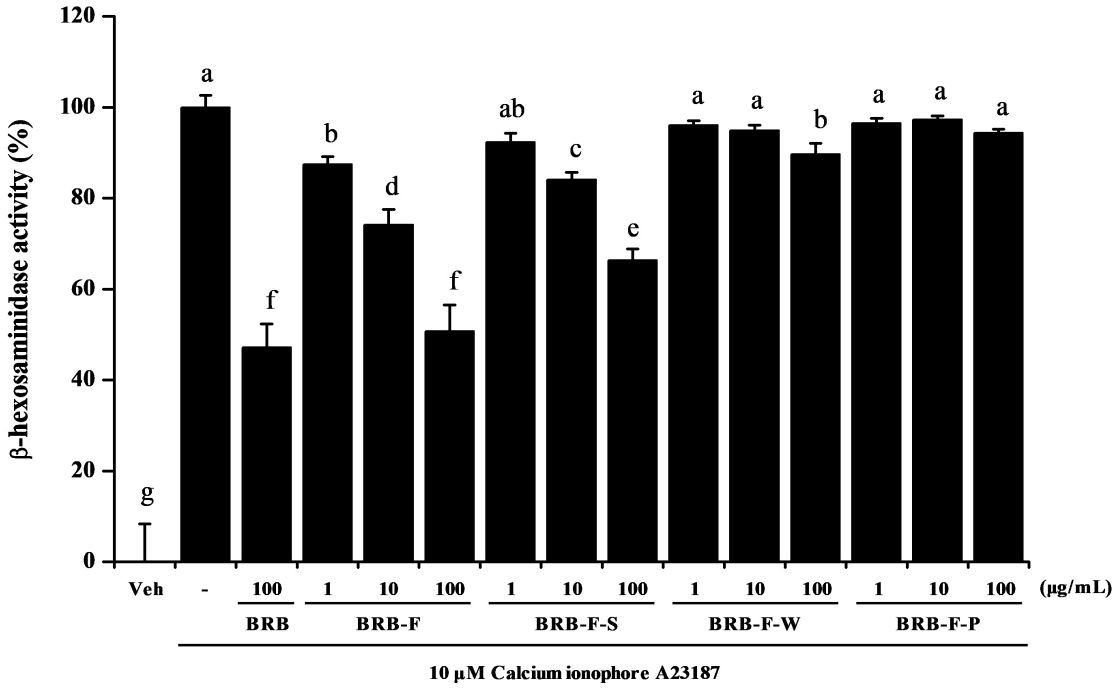

Figure 5. Inhibitory effect of BRB-F and its isolated fractions on mast cell activation (degranulation) RBL-2H3 mast cells were stimulated for 20 minutes with A23187 after each sample treatment for 15 minutes, and then the amount of β-hexosaminidase in the supernatant was measured. Data are expressed as mean ± SD (n = 3). Bars sharing a common letter are not significantly different between groups at $p < 0.05$.

by stimulation with LPS and IL-4 in the human B cell line U266.B1 was investigated. BRB-F and the further purified fractions were utilized (1 - 100 μg/mL) to treat U266.B1 cells for 72 h, then the amount of IgE in culture medium was measured using the ELISA method. LPS and IL-4 stimulation increased the amount of IgE 47-fold (508.2 ± 68.1 ng/mL) in U266.B1 cells compared to vehicle (10.7 ± 2.3 ng/mL), and treatment with 100 μg/mL BRB inhibited IgE production by 11%. When the BRB-F samples were added at 1 - 100 μg/mL, a dose-dependent inhibitory effect was observed, and a difference in the inhibitory effect, according to the recovery rate of the isolated fraction, was also observed (Figure 6).

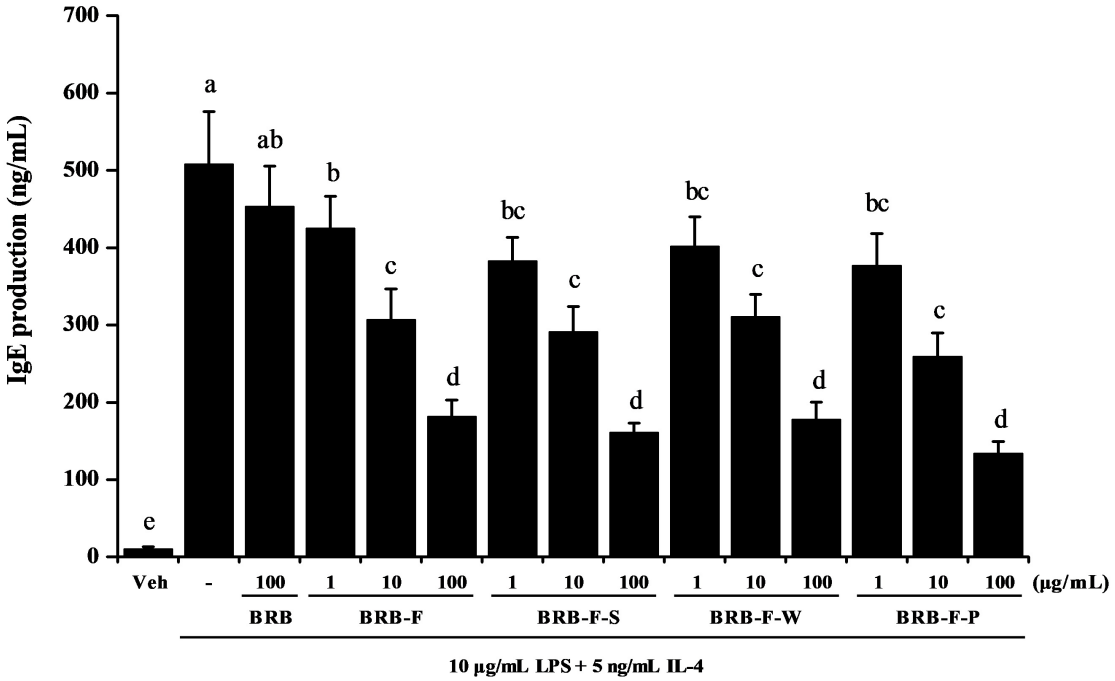

Figure 6. Inhibitory effect of BRB-F and its isolated fractions on IgE production in B cells. U266.B1 cells, each sample was treated with LPS (10 μg/mL) and IL-4 (5 ng/mL) for 72 h, and the amount of IgE was measured by ELISA by collecting the supernatant. Data are expressed as mean ± SD (n = 3). Bars sharing a common letter are not significantly different between groups at $p < 0.05$.

Note that because sensitization and stimulation are required to occur at the same time, a single stimulus by LPS or IL-4 alone will not activate the cells.

3.4. Inhibition of Chemokine Production in BEAS-2B Human Bronchial Epithelial Cells by BRB-F-S

To evaluate the inhibition by BRB-F-S (10 μg/mL) of the production of chemokines secreted upon treatment with TNF-*α* alone or IL-4 and TNF-*α* combined in BEAS-2B human bronchial epithelial cells, the amount of chemokine (RANTES, eotaxin or eotaxin-3) secreted into the culture medium was measured using the ELISA method. In BEAS-2B cells, treatment with TNF-*α* alone resulted in a 19-fold increase in the amount of RANTES (277.0 ± 22.3 pg/mL) compared to vehicle (14.5 ± 4.4 pg/mL), and pre-treatment with 10 μg/mL of BRB-F-S inhibited this stimulation by 43% (164.2 ± 4.0 pg/mL). In addition, the increases in eotaxin and eotaxin-3 upon treatment with IL-4 + TNF-*α* were inhibited by 58% and 21%, respectively, by pre-treatment with BRB-F-S (Figure 7).

3.5. Inhibition of OVA-Specific IgE Production in BALF

In the OVA-induced asthma mouse model, the inhibitory effect on OVA-specific IgE production in BALF by the administration of BRB-F and the isolated fractions was confirmed using the ELISA method. The amount of OVA-specific IgE in the BALF of the OVA-only group was increased 25-fold (175.3 ± 11.7 ng/mL) compared to the normal group (7.1 ± 0.6 ng/mL). In the group administered

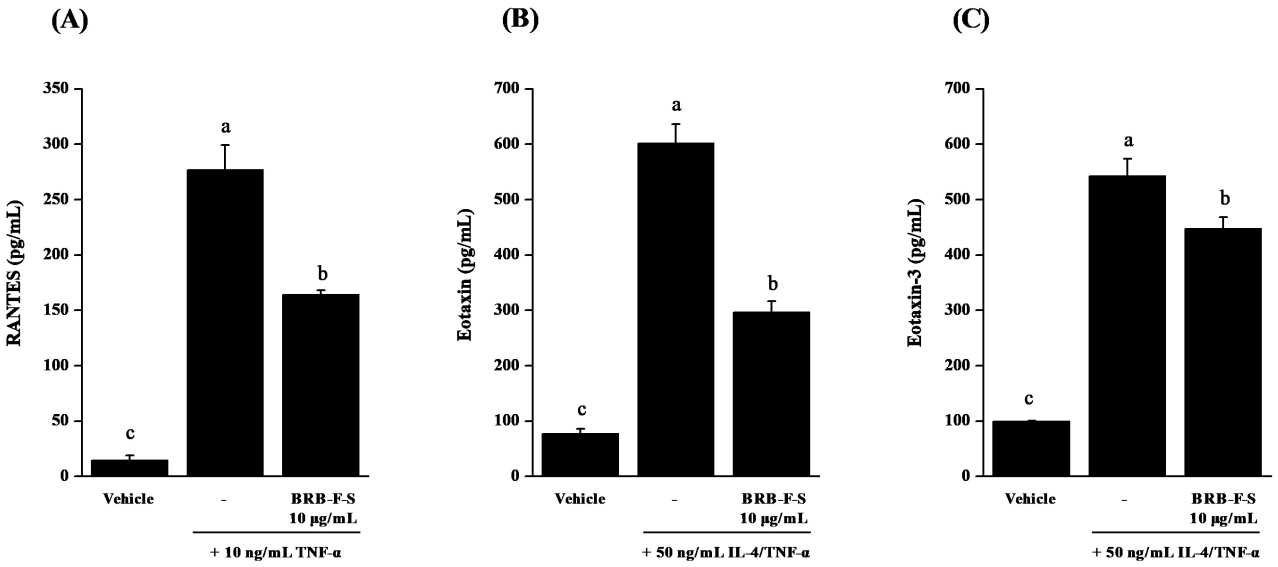

Figure 7. Inhibitory effect of BRB-F-S on the inflammatory reaction in bronchial epithelial cells. BEAS-2B bronchial epithelial cells were treated with BRB-F-S for 48 hours, and then stimulated by treatment with TNF-α or IL-4 + TNF-α. Thereafter, the amounts of RANTES, eotaxin, and eotaxin-3 in the supernatant were measured by ELISA. Data are expressed as mean ± SD (n = 3). Bars sharing a common letter are not significantly different between groups at $p < 0.05$.

with 40 mg/kg of BRB and BRB-F, the inhibition was 13% and 60%, respectively. Considering the recovery rate according to sequential purification, when BRB-F-S, BRB-F-W, and BRB-F-P were administered at a dose corresponding to 40 mg/kg of BRB-F, 70%, 67%, and 69% inhibition was shown, respectively (Figure 8). Note that only OVA-specific IgE was measured using the OVA-specific IgE ELISA kit, not total IgE.

3.6. Inhibition of OVA-Specific IgG Isotype (IgG, IgG1, IgG2a) Production in Serum

In the OVA-induced asthma mouse model, the ability of BRB-F and isolated fractions to inhibit the production of OVA-specific IgG isotypes (IgG, IgG1, IgG2a) in serum was confirmed. Asthma-induced mice showed a 51-fold, 34-fold, and 4-fold increase in total IgG, IgG1, and IgG2a levels, respectively, compared to the normal group. Mice administered with BRB and BRB-F at 40 mg/kg showed 13% and 58% inhibition, respectively, of total IgG production. Considering the recovery rate according to sequential purification, when BRB-F-S, BRB-F-W, and BRB-F-P were administered at a dose corresponding to 40 mg/kg of BRB-F, 63%, 53%, and 61% inhibition was seen, respectively (Figure 9). IgG1 production was inhibited by 9%, 61%, 71%, 54%, and 64% (BRB, BRB-F, BRB-F-S, BRB-F-W and BRB-F-P, respectively), and IgG2a production was inhibited by 36%, 39%, 32%, 29%, and 36%, respectively.

As a result of measuring the total amount of IgG, IgG1, and IgG2a in serum and comparing the activation of the Th1/Th2 immune response, when BRB-F and its purified fractions were administered, they had an excellent inhibitory effect on the production of total IgG and IgG1 compared with BRB. In contrast,

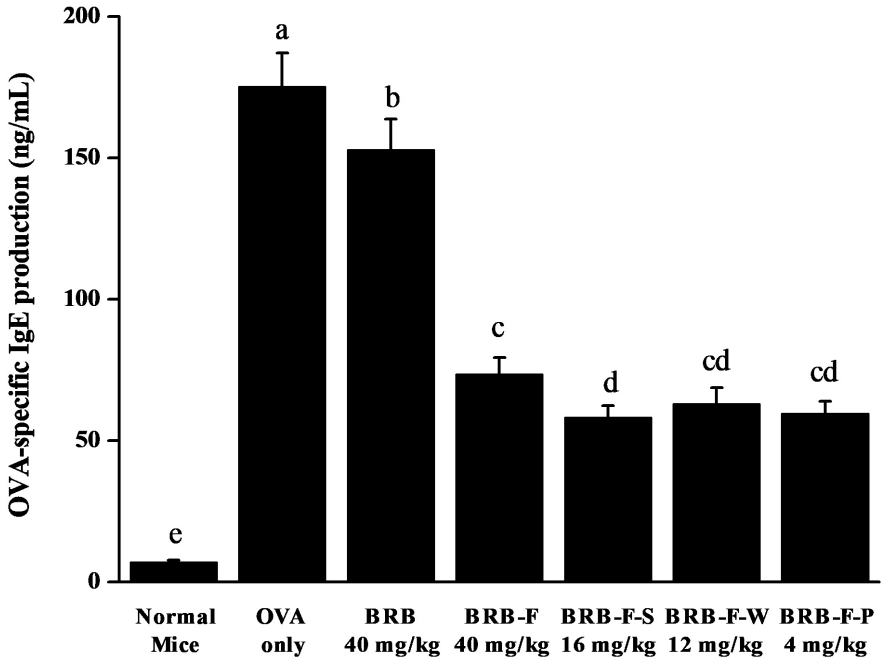

Figure 8. The OVA-specific IgE level in bronchoalveolar lavage fluid (BALF) from the OVA-induced asthma mouse model orally administered with BRB, BRB-F, and its purified products. BALF was collected from mice by lavaging tracheas with PBS. After centrifugation, supernatant was recovered from each mouse group and the OVA-specific IgE level in BALF was quantified using the ELISA kit. Data are expressed as mean ± SD (n = 10). Bars sharing a common letter are not significantly different between groups at $p < 0.05$.

the inhibitory effects of BRB-F and isolated fractions on IgG2a production were almost the same as those of BRB. In summary, the inhibitory effect on IgG1 generated by the Th2 immune response is greater than the inhibitory effect on IgG2a generated by the Th1 immune response. Only the fraction of OVA-specific isotypes (IgG, IgG1, and IgG2a), not total, was measured.

3.7. Effect of BRB-F and Its Isolated Fractions on Secretion of Th1, Th2, and Treg Cytokines

In a mouse model of OVA-induced asthma, the effects of administering BRB-F and its isolated fractions on Th1, Th2, and Treg cytokines secreted in BALF and serum were confirmed. The amounts of the Th2 cytokines IL-4, IL-5, and IL-13 in the BALF of the asthma-induced mice were around, or more than, 10-fold greater than those in the BALF of normal mice. The production of all three cytokines was inhibited by about 10% in the group administered with 40 mg/kg of BRB, and by more than 50% in the group administered with BRB-F. The cytokines were also inhibited by 50% or more in the groups administered with the isolated fractions (BRB-F-S, BRB-F-W and BRB-F-P) at a dose corresponding to 40 mg/kg BRB-F (Table 5). The amounts of the Th1 cytokines IL-2 and IL-12 in the serum of the asthma-induced mice was 33% - 50% lower than that of the normal mice. The amounts of IL-2 and IL-12 were recovered by 15% and 24%,

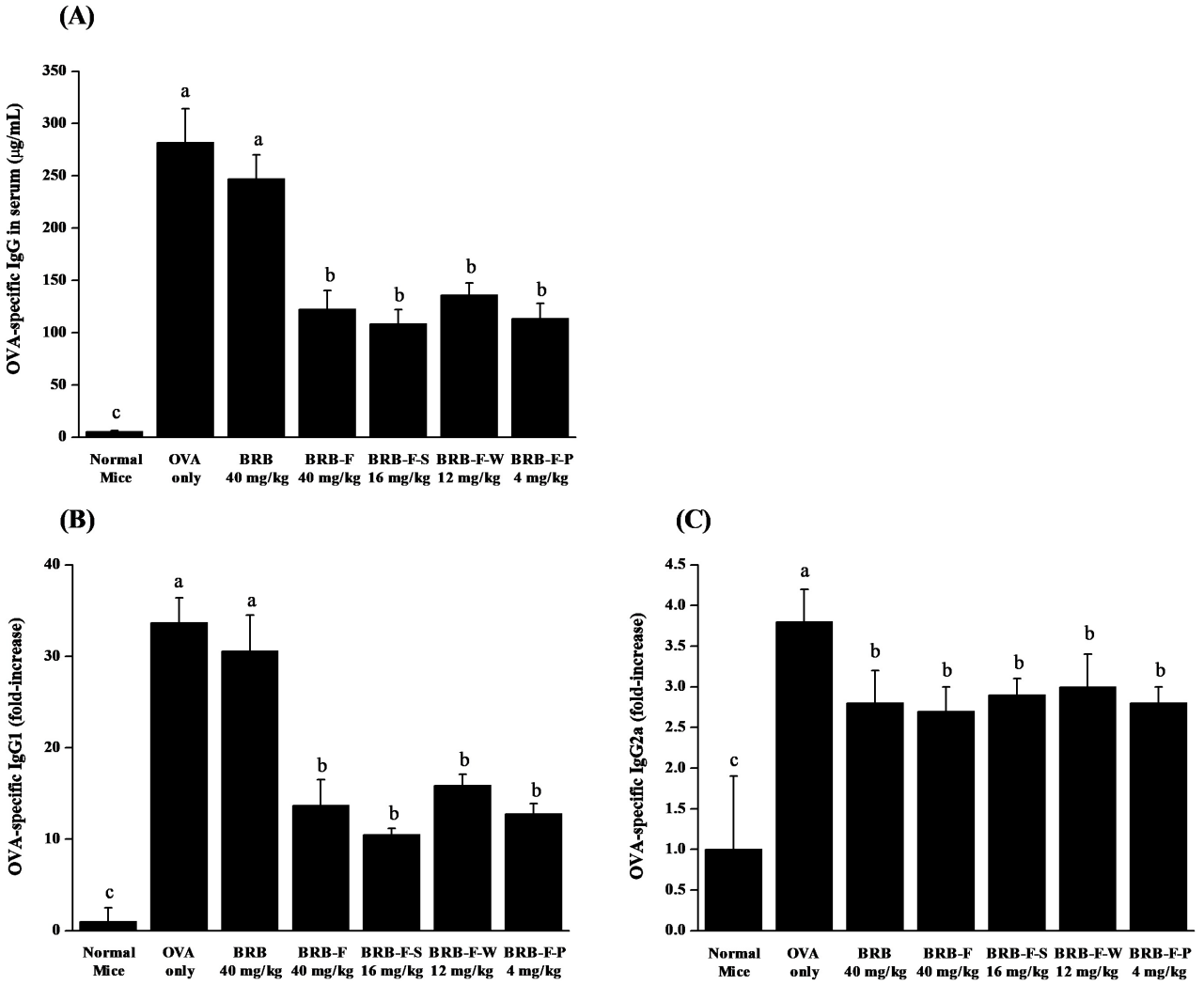

Figure 9. OVA-specific IgG, IgG1, and IgG2a level in serum from OVA-induced asthma mouse model orally administered with BRB, BRB-F, and its purified products. Blood was collected by cardiac puncture and serum was collected after blood clotting reaction. The resultant supernatant was quantitatively assayed for IgG, IgG1, and IgG2a levels of each mouse group using the ELISA method. Data are expressed as mean ± SD (n = 10). Bars sharing a common letter are not significantly different between groups at $p < 0.05$).

Table 5. Effects of BRB and its isolated fractions on Th1 and Th2 cytokines.

	Cytokine production (ng/mL)					
	BALF			Serum		
	IL-4	IL-5	IL-13	IL-2	IL-12	IL-10
Vehicle	8.3 ± 0.6^{c}	10.7 ± 0.8^{c}	11.2 ± 0.9^{c}	18.3 ± 1.5^{a}	252.2 ± 20.7^{a}	98.3 ± 8.7^{a}
OVA only	81.5 ± 7.2^{a}	132.9 ± 11.2^{a}	138.2 ± 10.7^{a}	10.5 ± 0.9^{c}	173.6 ± 15.3^{d}	51.2 ± 4.3^{d}
BRB (40 mg/kg)	75.9 ± 4.8^{a}	118.9 ± 8.9^{a}	115.1 ± 10.5^{a}	11.7 ± 0.8^{c}	192.4 ± 9.8^{c}	66.7 ± 4.2^{c}
BRB-F (40 mg/kg)	44.8 ± 3.5^{b}	76.2 ± 5.4^{b}	71.5 ± 5.8^{b}	15.2 ± 1.1^{b}	213.7 ± 17.2^{b}	76.9 ± 5.6^{b}
BRB-F-S (16 mg/kg)	38.1 ± 2.9^{b}	62.4 ± 3.9^{c}	60.9 ± 5.9^{b}	16.3 ± 1.3^{ab}	238.5 ± 10.5^{b}	82.3 ± 7.2^{b}
BRB-F-W (12 mg/kg)	40.6 ± 3.3^{b}	66.8 ± 5.6^{bc}	65.4 ± 6.1^{b}	15.8 ± 1.4^{ab}	226.7 ± 16.4^{b}	77.5 ± 5.9^{b}
BRB-F-P (4 mg/kg)	40.2 ± 2.7^{b}	63.6 ± 2.7^{c}	63.2 ± 4.7^{b}	16.0 ± 1.5^{ab}	229.4 ± 14.8^{b}	79.1 ± 6.1^{b}

Values in each column with the same superscript letters are not significantly different between groups at $p < 0.05$.

respectively, in the group administered with 40 mg/kg of BRB and by 60% and 51%, respectively, in the group administered with BRB-F. The levels of IL-2 and IL-12 in the groups administered with the isolated fractions at a dose corresponding to 40 mg/kg BRB-F also recovered more than 60%. The amount of IL-10 in serum as Treg cytokine was reduced 50% by asthma induction compared to that in the normal group. The group administered with BRB and BRB-F recovered 33% and 55%, respectively, and the group administered with the isolated fractions of BRB-F also recovered more than 60%.

3.8. Effect of BRB-F and Its Isolated Fraction on Immune Cell Infiltration in BALF

The effect of administration of BRB-F and its isolated fraction on the immune cell population in BALF in the OVA-induced asthma mouse model was determined. The total number of immune cells in BALF increased 5-fold ($1.62 \pm 0.11 \times 10^6$ cells) upon induction of OVA compared to the normal group ($0.34 \pm 0.02 \times 10^6$ cells). Administration of 40 mg/kg of BRB and BRB-F reduced the total number of immune cells in BALF by 11% and 39%, respectively. The group administered the isolated fractions of BRB-F showed a reduction effect of 40% or more (**Figure 10**). In addition, the cell types (lymphocyte, neutrophil, macrophage, and eosinophil) present in the BALF were measured. For asthma-induced mice, it was confirmed that the number of these immune cells was increased 12-, 9-, 4-, and 49-fold, respectively, compared to those in normal mice. Infiltration by these immune cells was also found to be inhibited by the administration of BRB-F and its isolated fractions. The data are based on specific, not overall, inhibition.

3.9. Effect of BRB-F and Its Isolated Fractions on Relief of Inflammation in Lung Tissue

To evaluate the anti-inflammatory effect of BRB-F and its isolated fractions in the lung tissue of a mouse model of OVA-induced asthma, cells were stained with hematoxylin & eosin Y. As asthma was induced by OVA, it was confirmed that the infiltration of immune cells in the lung tissue increased compared to the normal group. Conversely, it was found that BRB, BRB-F and its isolated fractions had a protective effect on lung tissue by suppressing immune cell infiltration and reducing inflammation (**Figure 11**).

3.10. Effect of BRB-F and Its Isolated Fractions on Relief of Inflammation in Lung Tissue

The levels of chemoattractants (eotaxin, vascular cell adhesion molecule-1 (VCAM-1)) and eicosanoids (leukotriene-4 (LTC_4), prostaglandin D2 (PGD_2)) that indicate airway inflammation were confirmed using the ELISA method. As asthma was induced by OVA, the amounts of all four inflammatory mediators increased in BALF compared to those in the normal group. There was an inhibitory effect on all inflammatory mediators of approximately 25% or more in the

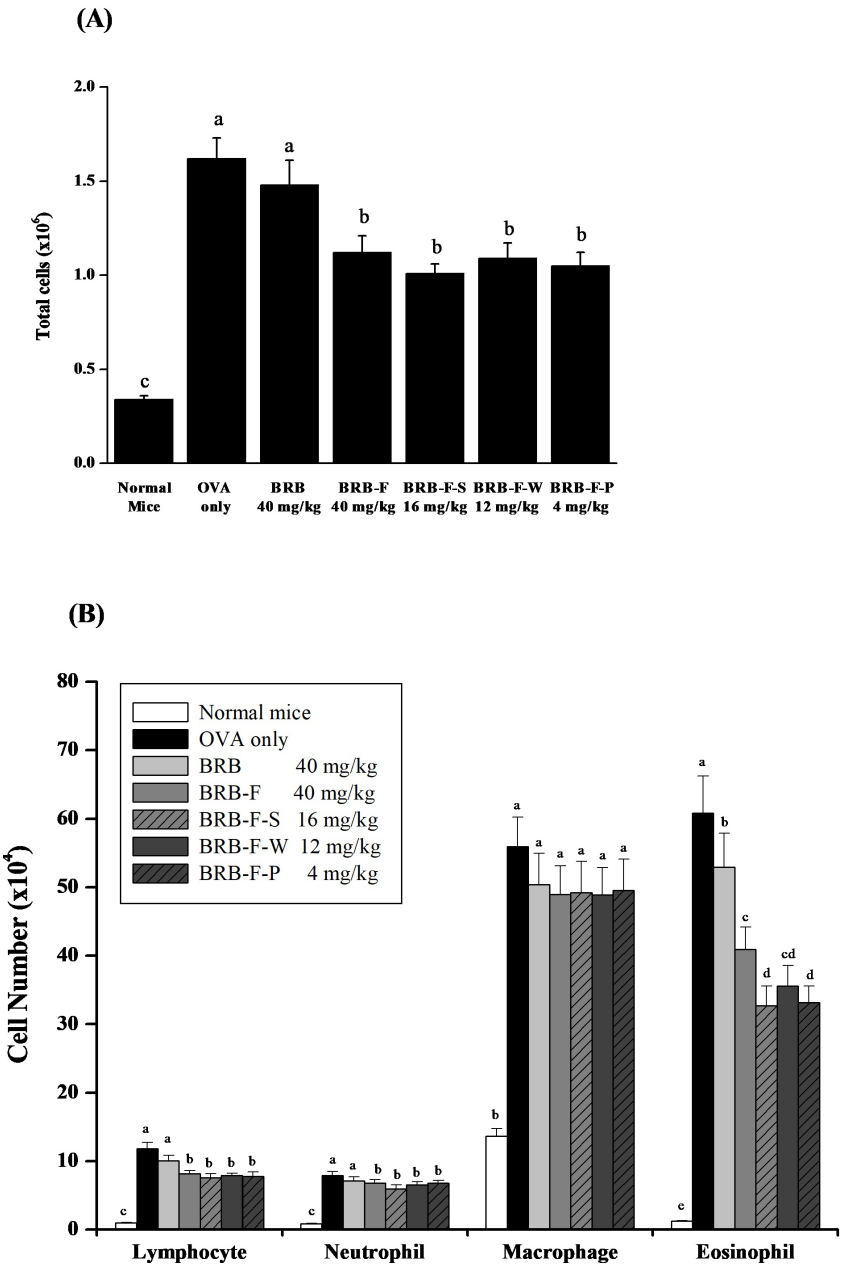

Figure 10. Inhibitory effect of BRB-F and its isolated fractions on immune cell infiltration in OVA-induced asthma mouse model. BALF was collected and centrifuged to obtain cell pellets, which were resuspended in PBS, followed by centrifuging onto slide glass and subsequent staining with Wright-Giemsa staining. The slides were microscopically observed (magnification, ×40) for differential cell count by counting a total of 300 cells per slide (B). Total cell number in BALF was measured by cell counting using a hemocytometer (A). Data are expressed as mean ± SD (n = 10). Bars sharing a common letter are not significantly different between groups at $p < 0.05$.

group administered with 40 mg/kg of BRB, and an inhibitory effect of approximately 70% in the BRB-F group. The administration of all the isolated fractions of BRB-F resulted in an inhibitory effect of 70% or more on all inflammatory mediators, confirming an inhibitory effect similar to that of BRB-F (Table 6).

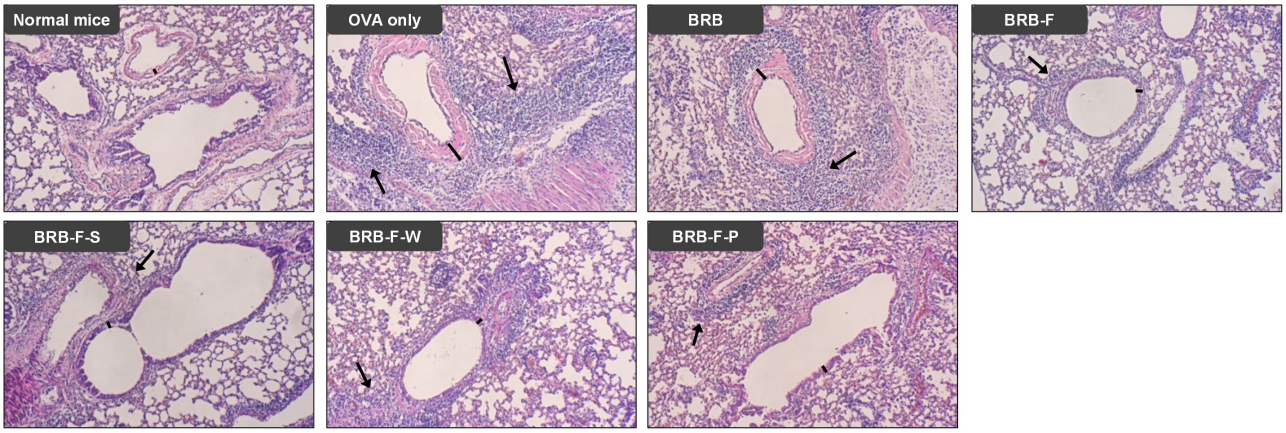

Figure 11. Example inhibitory effect of BRB-F and its isolated fractions on lung inflammation in an OVA-induced asthma mouse model. Lung tissues from mice were harvested and fixed with 10% (v/v) paraformaldehyde. The fixed tissues were sectioned to 4 μm, followed by staining with hematoxylin and eosin Y (H&E) and light microscopy (magnification, ×100). The black arrows indicate the aggregation of inflammatory cells, and the black vertical line indicates the airway wall thickness. Arrows indicate the level of tracheal edema that resulted from inflammatory cell infiltration in lung tissue from each mouse group. Figures represent the average of at least three individual repetitions).

Table 6. Effects of BRB and its isolated fractions on chemoattractant and eicosanoid production.

	Chemoattractants (pg/mL)		Eicosanoids (ng/mL)	
	Eotaxin	VCAM-1	LTC4	PGD2
Vehicle	32.7 ± 3.7^{d}	3.5 ± 0.2^{e}	47.2 ± 4.3^{c}	31.4 ± 2.8^{d}
OVA only	139.4 ± 10.9^{a}	28.7 ± 2.4^{a}	92.5 ± 5.9^{a}	72.9 ± 6.9^{a}
BRB (40 mg/kg)	112.8 ± 8.7^{b}	21.6 ± 2.5^{b}	82.5 ± 6.7^{a}	55.8 ± 5.5^{b}
BRB-F (40 mg/kg)	63.6 ± 7.2^{c}	15.7 ± 1.9^{c}	56.8 ± 5.5^{b}	42.7 ± 3.7^{c}
BRB-F-S (16 mg/kg)	54.7 ± 6.8^{c}	12.4 ± 1.4^{d}	47.2 ± 5.3^{c}	41.9 ± 4.8^{c}
BRB-F-W (12 mg/kg)	60.9 ± 5.4^{c}	12.9 ± 0.9^{d}	53.9 ± 6.4^{b}	44.9 ± 3.1^{c}
BRB-F-P (4 mg/kg)	55.4 ± 6.2^{c}	13.5 ± 1.5^{cd}	51.4 ± 4.7^{bc}	40.3 ± 3.9^{c}

Values in each column with the same superscript letters are not significantly different between groups at $p < 0.05$.

3.11. Effect on Inflammatory Cytokines and Chemokines in BALF

The effect of the administration of BRB-F on the production of inflammatory cytokines and chemokines in the BALF in a mouse model of OVA-induced asthma was evaluated. As asthma was induced by OVA induction, the amount of the three inflammatory cytokines TNF-α, IL-1β, and IL-6 increased compared to the normal group. For example, TNF-α increased three-fold (73.5 ± 5.7 pg/mL) in the asthma-induced group compared to levels in the normal group (27.7 ± 3.6 pg/mL). The administration of 40 mg/kg BRB inhibited the amount of TNF-α in BALF by 6%, and BRB-F inhibited TNF-α in BALF by 76%. TNF-α was inhibited by 70% or more in the groups administered with the isolated fractions (at a dose corresponding to 40 mg/kg BRB-F). IL-1β and IL-6 were also reduced by BRB-F

and the isolated fractions (Table 7). In addition, as asthma was induced by OVA, the level of the chemokine CXCL1 in BALF increased about three-fold (209.7 ± 19.5 pg/mL) compared to that in the normal group (82.5 ± 7.6 pg/mL), and administration of BRB, BRB-F and isolated fractions BRB-F-S, BRB-F-W and BRB-F-P inhibited the increase in CXCL1 by 16%, 53%, 64%, 57%, and 52%, respectively.

3.12. Inhibition of Immunoglobulin Production in Relation to the Dose of BRB-F-S

To confirm the relationship between the asthma inhibitory effect and the dose of BRB-F-S, the immunoglobulin production inhibitory effect was determined at four different doses. The dose of BRB-F-S was 20, 40, 80, and 160 mg/kg BRB-F equivalents, in consideration of the recovery rate compared to BRB-F. The amount of OVA-specific IgE, IgG, IgG1, and IgG2a increased compared to the normal group upon asthma induction but decreased at a level dependent on the concentration of BRB-F-S administered. When BRB-F-S at the maximum dose of 64 mg/kg was administered, the inhibitory effects were 68%, 71%, 81%, and 55% for IgE, IgG, IgG1, and IgG2a, respectively (Figure 12).

3.13. Effect of BRB-F-S on Secretion of Th1, Th2, and Treg Cytokines in Relation to Dose

The levels of Th2 cytokines (IL-4, IL-5, IL-13) present in the BALF from the OVA-induced asthma mouse model were shown to increase on asthma induction compared to the normal group. As the dose of BRB-F-S administered to the mice increased, greater inhibition of Th2 cytokine production was observed. When BRB-F-S was administered at the maximum dose of 64 mg/kg, the levels of IL-4, IL-5, and IL-13 were inhibited by 66%, 56%, and 62%, respectively (Table 8). The levels of Th1 cytokines and Treg cytokine (IL-2, IL-12, and IL-10)

Table 7. Effects of BRB and its isolated fractions on pro-inflammatory cytokine and chemokine production.

	Pro-inflammatory cytokine (pg/mL)			Chemokine (pg/mL)
	TNF-α	IL-1β	IL-6	CXCL1
Vehicle	27.7 ± 3.6^{c}	31.5 ± 4.4^{c}	19.7 ± 2.6^{c}	82.5 ± 7.6^{c}
OVA only	73.5 ± 5.7^{a}	59.8 ± 6.9^{a}	42.4 ± 3.7^{a}	209.7 ± 19.5^{a}
BRB (40 mg/kg)	70.9 ± 6.8^{a}	55.7 ± 5.1^{a}	40.9 ± 4.5^{a}	189.4 ± 21.7^{a}
BRB-F (40 mg/kg)	38.5 ± 4.2^{b}	39.4 ± 4.5^{b}	25.8 ± 3.3^{b}	141.7 ± 16.8^{b}
BRB-F-S (16 mg/kg)	36.9 ± 4.9^{b}	40.2 ± 3.7^{b}	23.9 ± 3.1^{b}	128.9 ± 10.7^{b}
BRB-F-W (12 mg/kg)	41.7 ± 5.8^{b}	36.8 ± 4.8^{b}	27.8 ± 2.9^{b}	137.5 ± 15.6^{b}
BRB-F-P (4 mg/kg)	35.4 ± 4.1^{b}	39.7 ± 5.1^{b}	25.9 ± 3.5^{b}	143.4 ± 17.4^{b}

Values in each column with the same superscript letters are not significantly different between groups at $p < 0.05$.

Table 8. Dose-dependent effects of BRB-F-S on Th1 and Th2 cytokines.

	Cytokine production (ng/mL)					
	BALF			Serum		
	IL-4	IL-5	IL-13	IL-2	IL-12	IL-10
Vehicle	18.5 ± 2.1^{e}	16.3 ± 1.5^{d}	15.9 ± 1.3^{e}	27.8 ± 3.1^{a}	253.7 ± 22.6^{a}	93.4 ± 8.5^{a}
OVA only	107.6 ± 8.9^{a}	151.5 ± 13.7^{a}	163.7 ± 15.1^{a}	7.9 ± 0.8^{e}	168.9 ± 14.1^{c}	48.2 ± 3.7^{e}
BRB-F-S (8 mg/kg)	77.6 ± 8.5^{b}	113.6 ± 10.9^{b}	111.7 ± 12.9^{b}	14.3 ± 1.5^{d}	182.8 ± 20.8^{bc}	60.6 ± 5.9^{d}
BRB-F-S (16 mg/kg)	60.3 ± 5.4^{c}	86.5 ± 9.5^{c}	89.3 ± 9.5^{c}	18.1 ± 1.3^{c}	196.3 ± 21.6^{b}	75.4 ± 6.4^{c}
BRB-F-S (32 mg/kg)	54.8 ± 4.7^{cd}	80.5 ± 8.3^{c}	77.5 ± 7.2^{cd}	20.3 ± 2.2^{bc}	205.7 ± 17.2^{b}	81.7 ± 7.2^{bc}
BRB-F-S (64 mg/kg)	48.9 ± 5.1^{d}	75.9 ± 6.8^{c}	71.8 ± 5.6^{d}	22.7 ± 1.7^{b}	216.3 ± 15.6^{b}	88.9 ± 8.9^{b}

Values in each column with the same superscript letters are not significantly different between groups at $p < 0.05$.

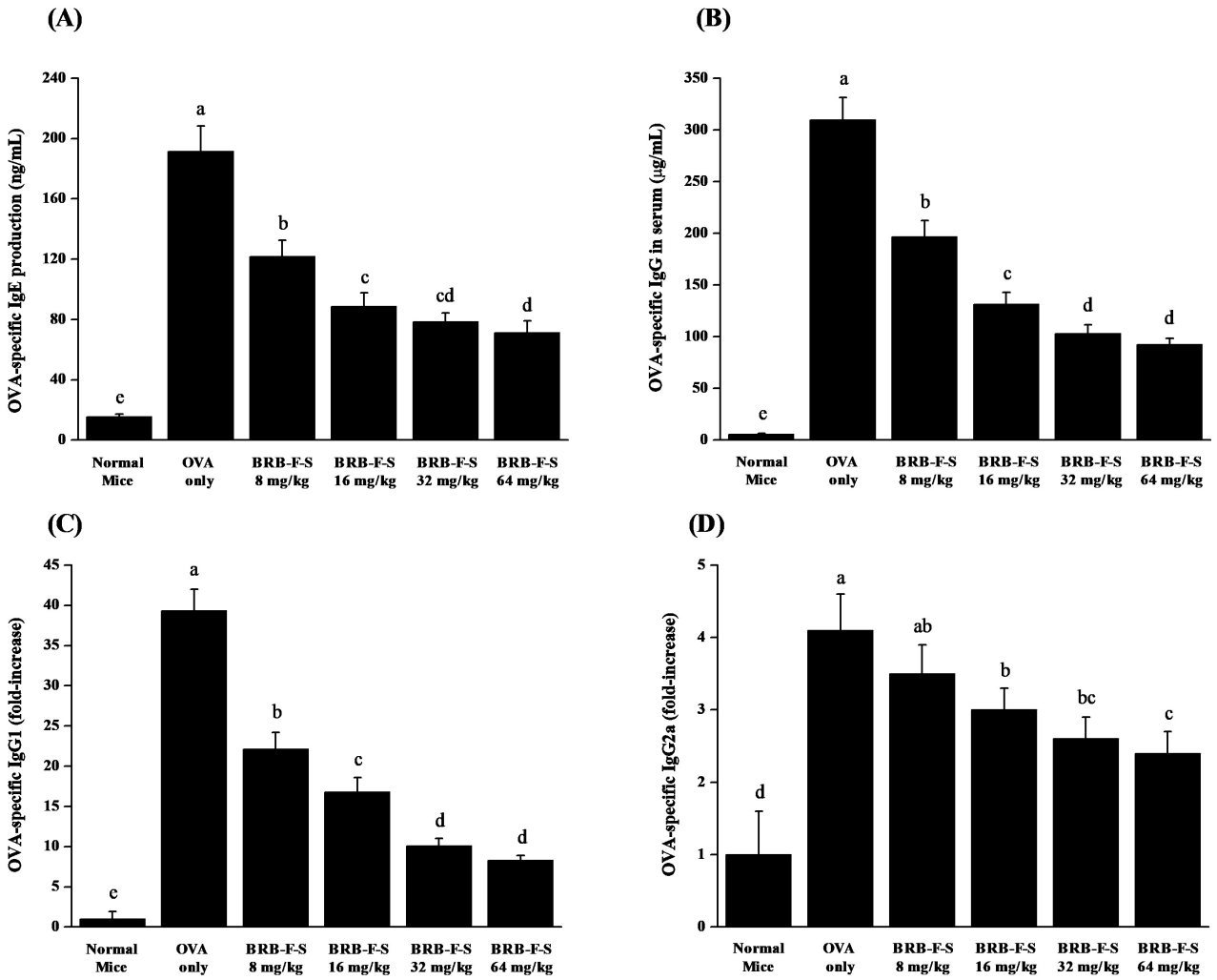

Figure 12. Dose-dependent inhibitory effect of BRB-F-S on immunoglobulin production in the OVA-induced asthma mouse model. BALF was collected from mice by lavaging tracheas with PBS. After centrifugation, supernatant was recovered from each mouse group and OVA-specific IgE level was quantified in BALF using the ELISA kit (A). Blood was collected by cardiac puncture and serum was collected after blood clotting reaction. The resultant supernatant was quantitatively assayed for IgG, IgG1, and IgG2a levels of each mouse group using the ELISA method (B-D). Data are expressed as mean ± SD (n = 10). Bars sharing a common letter are not significantly different between groups at $p < 0.05$.

present in the serum were also measured: all three were reduced by asthma induction and recovered by administering BRB-F-S in a dose-dependent manner. When BRB-F-S was administered at the maximum dose of 64 mg/kg, the amounts of IL-2, IL-12, and IL-10 were recovered by 74%, 56%, 90%, respectively.

3.14. Effect of BRB-F-S on Immune Cell Infiltration and Lung Inflammation in Relation to Dose

The effect of BRB-F-S on the total number of immune cells and cell population in BALF from the OVA-induced asthma mouse model was confirmed. The total number of immune cells increased upon asthma induction, compared to the normal group, and, as the dose of BRB-F-S administered increased, the inhibition of infiltration by immune cells in BALF also increased (**Figure 13**). This was confirmed by staining with hematoxylin & eosin Y to evaluate the anti-inflammatory effect in the lung tissue, in which the infiltration of immune cells in the lung tissue was increased by asthma induction. However, the infiltration was inhibited by BRB-F-S in a dose-dependent manner (**Figure 14**).

3.15. Dose-Dependent Effects of BRB-F-S on Chemoattractants and Eicosanoids

In the OVA-induced asthma mouse model, the amounts of chemoattractants (eotaxin, VCAM-1) and eicosanoids (LTC_4, PGD_2) in BALF were shown, using the ELISA method, to be increased by induction of asthma compared to the normal group. As the dose of BRB-F-S increased, the inhibitory effect on the production of chemoattractants and eicosanoids in BALF also increased. When BRB-F-S was administered at the maximum dose of 64 mg/kg, the production of eotaxin, VCAM-1, LTC_4, and PGD_2 was inhibited by 79%, 83%, 78%, and 93%, respectively (**Table 9**).

3.16. Dose-Dependent Effects of BRB-F-S on Chemoattractants and Eicosanoids

As asthma was induced, the amounts of inflammatory cytokines (TNF-α, IL-1β,

Table 9. Dose-dependent effects of BRB-F-S on chemoattractants and eicosanoids production.

	Chemoattractants (pg/mL)		Eicosanoids (ng/mL)	
	Eotaxin	VCAM-1	LTC4	PGD2
Vehicle	30.3 ± 3.2^{d}	3.1 ± 0.3^{f}	35.4 ± 2.9^{e}	35.9 ± 2.9^{d}
OVA only	141.9 ± 13.9^{a}	30.8 ± 2.7^{a}	112.7 ± 10.7^{a}	95.6 ± 8.1^{a}
BRB-F-S (8 mg/kg)	79.3 ± 5.6^{b}	20.3 ± 1.9^{b}	77.9 ± 5.8^{b}	65.8 ± 6.7^{b}
BRB-F-S (16 mg/kg)	$66.8 \pm 7.1b^{c}$	13.4 ± 1.4^{c}	66.7 ± 4.7^{c}	53.9 ± 5.5^{bc}
BRB-F-S (32 mg/kg)	59.6 ± 4.8^{c}	9.7 ± 0.8^{d}	59.1 ± 5.5^{cd}	46.8 ± 3.8^{c}
BRB-F-S (64 mg/kg)	54.2 ± 5.9^{c}	7.7 ± 0.6^{e}	52.5 ± 4.3^{d}	40.3 ± 3.9^{d}

Values in each column with the same superscript letters are not significantly different between groups at $p < 0.05$.

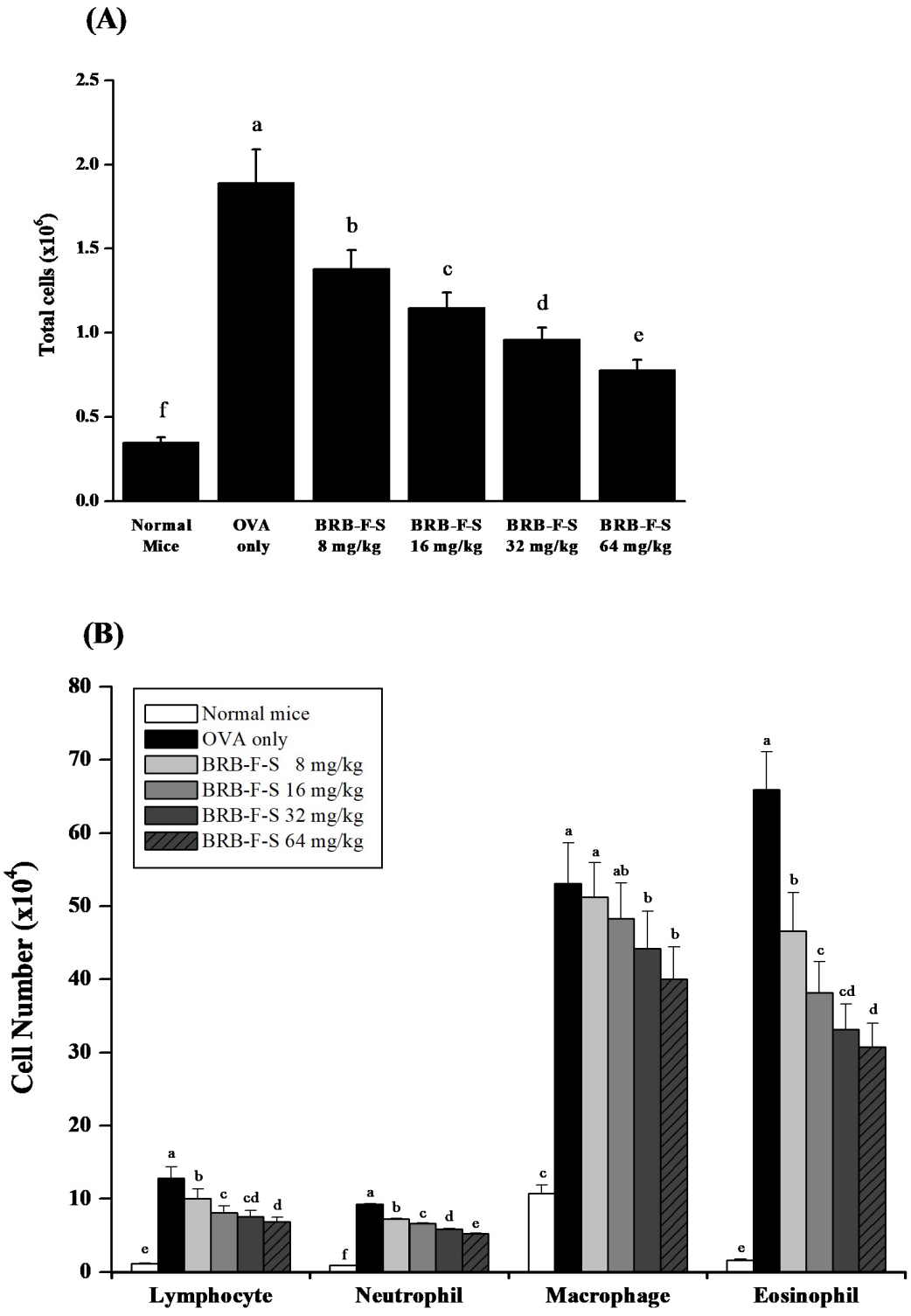

Figure 13. Dose-dependent inhibitory effect of BRB-F-S on immune cell infiltration in OVA-induced asthma mouse model. BALF was collected and centrifuged to obtain cell pellets, which were resuspended in PBS, followed by centrifuging onto slide glass and subsequent staining with Wright-Giemsa staining. The slides were microscopically observed (magnification, ×40) for differential cell count by counting a total of 300 cells per slide (B). Total cell number in BALF was measured by cell counting using a hemocytometer (A). Data are expressed as mean ± SD (n = 10). Bars sharing a common letter are not significantly different between groups at $p < 0.05$.

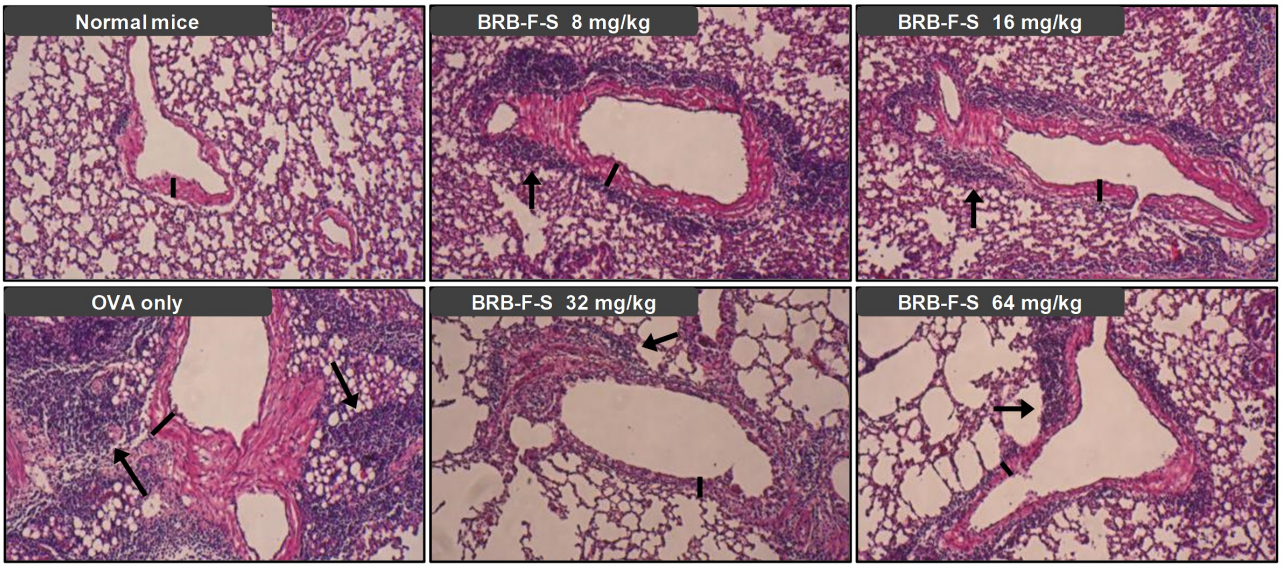

Figure 14. Dose-dependent inhibitory effect of BRB-F-S on lung inflammation in OVA-induced asthma mouse model. Lung tissues from mice were harvested and fixed with 10% (v/v) paraformaldehyde. The fixed tissues were sectioned to 4 μm, followed by staining with hematoxylin and eosin Y (H&E) and light microscopy (magnification, ×100). The black arrows indicate the aggregation of inflammatory cells, and the black vertical line indicates the airway wall thickness. Arrows indicate the level of tracheal edema that resulted from inflammatory cell infiltration in lung tissue from each mouse group. Figures represent the average of at least three individual repetitions.

IL-6) and chemokines (CXCL1) in BALF were increased compared to the normal group. However, as the dose of BRB-F-S increased, it was confirmed that the level of inflammatory cytokines and chemokines in BALF decreased in a dose-dependent manner. When BRB-F-S was administered at the maximum dose of 64 mg/kg, the production of TNF-α, IL-1β, IL-6, and CXCL1 was inhibited by 89%, 95%, 92%, and 86%, respectively (Table 10).

3.17. Dose-Dependent Inhibitory Effect of BRB-F-S on TSLP Production

The cytokine thymic stromal lymphopoietin (TSLP) induces a Th2 immune response by differentiating naive Th0 cells into Th2 cells, and it is mainly secreted from bronchial epithelial cells and skin keratinocytes at the initiation stage of allergic disease. Excessive secretion of TSLP induces a Th2 immune response to advance or aggravate allergic diseases, so the effect of BRB-F-S administration on TSLP secretion was investigated. There was a 2-fold (80.9 ± 7.6 pg/mL) increase in TSLP in the asthma induction group compared to the normal group (41.5 ± 3.8 pg/mL), and as the administered dose of BRB-F-S increased, so there was an increased inhibition of TSLP production. When BRB-F-S was administered at the maximum dose of 64 mg/kg, the inhibitory effect was 91% (Figure 15).

4. Discussion

The results presented in Figures 1-15 and Tables 1-10 of the present study show

Table 10. Dose-dependent effects of BRB-F-S on pro-inflammatory cytokines and chemokine production.

	Pro-inflammatory cytokine (pg/mL)			Chemokine (pg/mL)
	TNF-α	IL-1β	IL-6	CXCL1
Vehicle	21.9 ± 1.8^{f}	25.2 ± 2.4^{d}	15.9 ± 1.4^{e}	75.9 ± 5.8^{e}
OVA only	76.8 ± 5.9^{a}	61.1 ± 5.8^{a}	41.8 ± 3.5^{a}	236.2 ± 21.7^{a}
BRB-F-S (8 mg/kg)	50.4 ± 4.2^{b}	42.2 ± 1.9^{b}	30.1 ± 2.7^{b}	141.2 ± 13.5^{b}
BRB-F-S (16 mg/kg)	38.7 ± 2.1^{c}	32.4 ± 2.2^{c}	22.7 ± 2.1^{c}	118.9 ± 10.9^{c}
BRB-F-S (32 mg/kg)	32.6 ± 2.7^{d}	28.5 ± 2.1^{cd}	20.6 ± 2.2^{cd}	105.6 ± 7.8^{cd}
BRB-F-S (64 mg/kg)	27.8 ± 1.9^{e}	26.9 ± 1.6^{d}	18.1 ± 1.6^{d}	98.1 ± 6.4^{d}

Values in each column with the same superscript letters are not significantly different between groups at $p < 0.05$.

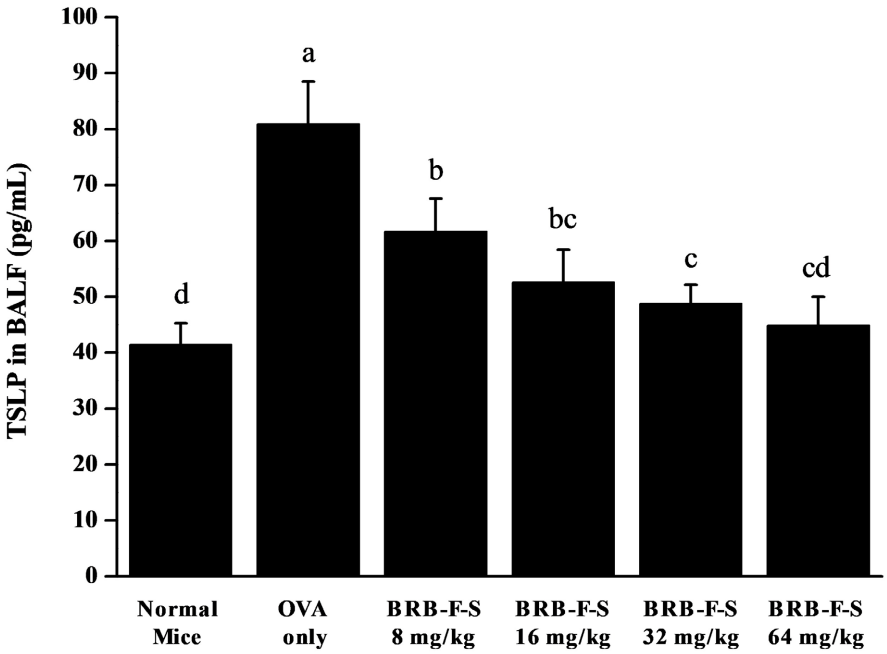

Figure 15. Dose-dependent inhibitory effect of BRB-F-S on TSLP production in the OVA-induced asthma mouse model. BALF was collected from mice by lavaging tracheas with PBS. After centrifugation, supernatant was recovered from each mouse group and the TSLP level in BALF was quantified using the ELISA kit. Data are expressed as mean ± SD (n = 10). Bars sharing a common letter are not significantly different between groups at $p < 0.05$.

that exposure of mast cells, B cells, and mice to the bioprocessed product and its purified fractions reduced the levels of multiple biomarkers that are known to cause the manifestations of allergic asthma. Specifically, in cell studies, the bioprocessed black rice bran/mushroom mycelia formulation (BRB-F) and three isolated fractions BRB-F-S, BRB-F-W, and BRB-F-P (Figure 1) inhibited mast cell activation, and also inhibited IgE production in U266.B1 cells. Therefore, the formulation might effectively control allergic diseases such as asthma via this in-

hibition. In addition, the fraction BRB-F-S inhibited the inflammatory reaction in BEAS-2B bronchial epithelial cells, as determined by changes in eotaxin and eotaxin-3. The inhibition of mast cell activation was decreased by the sequential purification process of BRB-F, but, until BRB-F-S, degranulation-inhibiting physiologically active ingredients remained, so it was judged that it would be possible to prevent and treat various allergic diseases by effectively inhibiting degranulation in mast cells.

In mouse models, changes were observed in OVA-specific IgE levels in bronchoalveolar lavage fluid (BALF) in response to BRB and the four bioprocessed samples, and in OVA-specific IgG, IgGl, and IgG2a in serum from asthma mice, and in terms of infiltration of lymphocytes, neutrophils, macrophages, and eosinophils. Administering each of the four bioprocessed samples inhibited lung inflammation in asthma mice, as determined by the histology of lung tissues. The BRB-F-S bioprocessed fraction was shown to have a dose-dependent inhibitory effect on immunoglobulin production in blood and BALF, on lung inflammation in mice, as determined by the histology of lung tissues, and on TSLP levels in BALF.

Other effects of the active products on asthma biomarkers include additional changes associated with the mechanism of asthma inhibition. For example, the inhibitory effects of BRB and the four bioprocessed samples on the production of the cytokines IL-4. IL-5, and IL-13 in BALF and IL-2, IL-12, and IL-10 in serum were observed; BRB and the four samples had an effect on the levels of the chemoattractants eotaxin and VCAM-1, the eicosanoids LTC_4 and PGD_2, and on pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 and chemokine CXCLl. The extract BRB-F-S had dose-dependent effects on the production of cytokines IL-4, IL-5, and IL-13 in BALF and IL-2, IL-12 in serum; on eotaxin, VCAM-1 LTC_4 and PGD_2; and on proinflammatory cytokines TNF-α, IL-lβ, and IL-6, and chemokine CXCLl.

The dose-dependent effects of the newly developed functional foods *in vitro* and *in vivo* reported here demonstrate the beneficial anti-asthma effects of these food products that need to be investigated further in human trials with asthmatic adults and children. The findings also enhance our understanding of the mechanism that seems to govern the anti-asthma effect in terms of changes in anti-inflammatory, immunochemical, and other biomarkers in BALF and serum, and the observed regeneration of lung tissues demonstrated by histology.

New functional foods obtained by bioprocessing black rice bran with mushroom mycelia may have additional health benefits. Colored (pigmented) rice flours are good sources of nutrients and biologically active compounds that include anthocyanins, flavonoids, and phenolic compounds. The multiple health benefits of these antioxidants are described elsewhere [13] [14]. Black rice bran also contains the bioactive compound γ-oryzanol, as previously reported [15] [16] [17], as well being confirmed in the present study. Based on the results, however, γ-oryzanol does not seem to significantly contribute to the anti-asthma effect of the fermented products because the level of γ-oryzanol decreases sequen-

tially through the purification steps, with no *γ*-oryzanol present in BRB-F-P, yet this fraction induced anti-asthma effects. From comparing all the asthma inhibitory effects of BRB-F and the isolated fractions, the BRB-F-derived polysaccharide was determined to be the most significant component of the inhibitory effect in the asthma mouse model. Indeed, it can be deduced that many of the inhibitory effects on asthma seem to be caused by BRB-F-P, which consists of a polysaccharide containing 96% carbohydrates, of which most are glucose and galactose (**Figure 4** and **Table 4**). However, the potency of BRB-F-S is somewhat higher than that of BRB-F-P, suggesting that both formulations have the potential to treat human asthma.

We previously reported that mushroom polysaccharides have the potential to help protect against multiple diseases [8] [9] [18]. Also relevant is that the composition of bioactive compounds in mushroom fruit bodies differs from those in mycelia of the same variety [19]. In addition, BRB-F-S induced a strong Th1 response, especially phagocytosis and phagocytic removal by the polysaccharide contained in BRB-F-S, suggesting that it is capable of inducing the rapid termination of an inflammatory response by inflammation-inducing compounds through phagocytosis. These considerations suggest that, in addition to anti-asthma properties, the newly developed functional foods might also help protect against other diseases or conditions, such as obesity, cancer, and diabetes, through these anti-inflammatory properties.

5. Conclusion

The results of the present study show that a new functional food created by bioprocessing black rice bran with shiitake mushroom mycelia inhibits mast cell degranulation, and B cell IgE production, and the pro-inflammatory effects seen in ovalbumin-induced asthma cell and mice models. The histology of lung tissues revealed that the treatment also reversed the thickening of the airway wall, the contraction of bronchial and blood vessels and infiltration of inflammatory cells. The novel food products may have useful therapeutic applications in humans. Future studies could therefore investigate if these new food products can protect humans against allergic asthma, and possibly also ameliorate the acute respiratory syndromes associated with other diseases or conditions, including viral infections such as COVID-19, chronic obstructive pulmonary disease (COPD), emphysema, as well as peanut protein and other food allergies.

Acknowledgements

We thank Alan D. Friedman (Johns Hopkins University Oncology) for his constructive comments.

Authors' Contributions

SPK conceived the idea with JK; KHL, KJK, KSK, SJL, WSH, and WYL did the experimental studies; MF and SPK interpreted the results and prepared a draft of

the manuscript. All authors read and approved the final manuscript.

Funding

This research was supported by the Technology Innovation Program (No. 20008826) funded by the Ministry of Trade, Industry & Energy (MOTIE, Korea).

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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