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RESEARCH NOTE

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Effects of γ-Irradiation on Immunological Activities of β-Glucan

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Abstract This study evaluated the effects of γ-irradiation on immunomodulating properties and structural changes of β-glucan. β-Glucan solutions (10 mg/mL) were γ-irradiated at 10, 30, and 50 kGy. Splenocyte proliferation and cytokine (interferon-γ and interlukin-2) productions by γ-irradiated β-glucan were evaluated in *in vivo* and *in vitro*, and structural changes of β-glucan were also determined after γ-irradiation. γ-Irradiation on β-glucan at 50 kGy enhanced splenocyte proliferation and cytokine productions, ($p<0.05$) and cleft glycosidic bonds of β-glucan resulting in lower the molecular weight. These results indicate that the use of γ-irradiation on β-glucan may be useful for improving its immunological activity by lowering the molecular weight of β-glucan.

Keywords: β-glucan, irradiation, depolymerization, glycosidic bond, immunological activity

Introduction

β-Glucan is composed of glucose units linked together to form a long polymer chain and is a fiber type derived from the cell walls of yeast, oat, barley, and many medicinal mushrooms (1). β-Glucans have immune-enhancing activities, which nutritionally potentiate and modulate an immune response (2). In general, degree of branching, polymer length, and tertiary structure of β-glucans influence their immunological activities (3). Several studies have reported correlations between immunological activities of β-glucan and molecular weight; low molecular weight β-glucans possess high immunological activities (4,5). Among the β-glucans lentinan (β-1,3-1,6-glucan) is the only one having low molecular weight and high immunological activities (6). The development of a method to lower the molecular weight of β-glucans would be very useful for immunological applications of β-glucans.

In order to decrease the molecular weight of β-glucan depolymerization methods by acidic (3,7) or enzymatic hydrolysis (8) have been used, but these methods have high cost, low yield, long processing time, and acidic wastes (9). γ-Irradiation would be a the method to overcome such disadvantages. γ-Irradiation also degrades polysaccharides such as starch, cellulose, and pectin by cleavage of the glycosidic bonds (10-16). Moreover, our previous study found that γ-irradiation improved solubility and decreased viscosity of β-glucan with no undesirable changes in functional groups (17). In addition, Charlesby (11) suggested that irradiation is a practical method to degrade polymers because it needs no chemical reagents and special equipments/setup to control the temperature, environment, and additives.

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The objective of this study was to evaluate the effects of γ-irradiation on the immunological activities of β-glucan and to determine structural changes of β-glucan after γ-irradiation.

Materials and Methods

Sample preparation and γ-irradiation β-Glucan samples were prepared according to the method published in a previous study (17). Briefly, powdered β-glucan (polysaccharide: 97.2%, total glucan: 80%; Ace Biotech, Ltd., Chungbuk, Korea) purified from black yeast (*Aureobasidium* species) was dissolved in deionized water to obtain a concentration of 100 mg/mL, and the solutions were γ-irradiated at 10, 30, and 50 kGy at room temperature and stored at 4°C until its use.

A cobalt-60 irradiator (point source AECL, IR-221, MDS Nordion International Co., Ltd., Ottawa, ON, Canada) at the Advanced Radiation Technology Institute of the Korea Atomic Energy Research Institute (Jeoungeup, Korea) was used for the γ-irradiation. The source strength was approximately 11.1 PBq with a dose rate of 10 kGy/hr. Dosimetry was carried out using a 5-mm diameter alanine dosimeter (Bruker Instruments, Rheinstetten, Germany). The dosimeter was calibrated using a standard of the International Atomic Energy Agency (Vienna, Austria).

Animals Six-week-old female BALB/c mice (body weight; 17-19 g) were purchased from Orient Inc. (Charles River Technology, Seoul, Korea). For vivarium adaption, the mice were housed in a polycarbonate cage and fed a standard animal diet and water *ad libitum* under controlled temperature conditions (22±2°C) with 12-hr light and dark cycles for a week before the β-glucan treatment.

Treatments and splenocyte suspension preparation To prepare a splenocyte suspension for the *in vitro* study, 5 mice were anesthesized with ether and sacrificed by

cervical decapitation, and the spleen was dissected and maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA). To prepare splenocytes, the spleen tissue was homogenized by glass homogenizer (Corning Inc., Lowell, MA, USA). Homogenates were then transferred to a sterile universal tube and centrifuged at 600×g for 5 min. The supernatant of the homogenate was discarded and the cell pellet was gently tapped to be resuspended. Two mL of sterile red blood cell lysis buffer (eBioscience Co., San Diego, CA, USA) was added to the cell suspension and incubated at room temperature for 1 min. For washing cells, 18 mL of RPMI 1640 medium was then added to the cell suspension and centrifuged at 600×g for 5 min, and the supernatant was removed. This washing step was repeated twice to wash out residuals of red blood cell lysis buffer and red blood cells to obtain a pellet of splenocytes. For the *in vivo* study, animals were divided into 5 groups (6 mice/group) that had been fed with (i) phosphate-buffered saline (PBS, Invitrogen Co., Carlsbad, CA, USA), (ii) unirradiated β-glucan (0 kGy), (iii) 10 kGy-, (iv) 30 kGy-, and (v) 50 kGy-irradiated β-glucan every day for 7 days. A concentration of 50 mg/kg body weight was provided the to animals by oral gavage (0.5 mL each). To obtain splenocyte suspensions from the mice fed with irradiated β-glucan and unirradiated β-glucan, these were prepared according to the same procedure as described above *in vitro* study.

Splenocyte proliferation assay The both splenocytes from *in vitro* and *in vivo* studies were resuspended in 10 mL of RPMI 1640 medium plus 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 units/mL streptomycin (complete medium), and cell viability (>90%) was then determined using trypan blue (18). Only for the *in vitro* study, splenocytes suspended in the complete medium were plated in 96-well tissue culture plates to reach a concentration of 1×10^6 cells/well. PBS, unirradiated (0 kGy), and irradiated β-glucans at 10, 30, and 50 kGy were then added to the cell suspension to have a concentration of 10 μg/mL of β-glucan and concanavalin A (1 μg/mL) was used as a mitogen. Splenocyte suspensions were then incubated at 37°C for 24 hr under 5% CO_2.

For the *in vivo* study, splenocytes suspension separated from the mice fed with unirradiated and irradiated β-glucan were plated in 96-well tissue culture plates without adding the β-glucan samples and concanavalin A (1 μg/mL) was just used as a mitogen followed by the incubation at 37°C for 24 hr under 5% CO_2. After incubation, splenocyte suspensions were centrifuged at 600×g for 5 min. The cell pellets were then used to determine splenocyte proliferation, and the supernatants were collected in microtubes and stored at −70°C to evaluate cytokine production.

Proliferation of splenocytes was detected using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) as described by Zhanga *et al*. (6). Briefly, 30 μL of MTT (5.0 mg/mL) dissolved in PBS was added into each well, and the plate was incubated at 37°C for 2 hr. After the plate was centrifuged at 600×g, the culture supernatants were removed, and 100 μL dimethyl sulfoxide (DMSO) was added to each well followed by incubation at 37°C for 5 min. Absorbance of samples was then measured at 595 nm using a microplate reader (Zenyth 3100; Anthos Labtec Instruments GmbH, Salzburg, Austria). The ratio of optical density (OD) value of no β-glucan added sample to OD of β-glucan added sample was calculated to represent the proliferation percentage.

Cytokine production The splenocyte suspensions were used for the cytokine production test. Enzyme-linked immunorbent assay (ELISA) kits (BD Biosciences, San Jose, CA, USA) were used with the splenocyte suspensions according to the manufacturer's instructions, and absorbance for interferon (IFN)-γ and interlukin (IL)-2 in splenocyte suspensions were measured at 450 nm with a microplate reader (Zenyth 3100). The absorbance values were then converted to concentrations (pg/mL) of IFN-γ and IL-2 using standard curves prepared with serial dilutions of IFN-γ and IL-2 standards.

Determination of structural changes of β-glucan

***Glycosidic bond linkage*:** After irradiation, the differences of the glycosidic bond (β-1,3 and β-1,6) lingkages between iradiated and unirradiated β-glucans were estimated with a β-D-glucan assay kit (Megazyme, Wicklow, Ireland) according to the manufacturer's procedure, and the glycosidic bonds of the irradiated and unirradiated β-glucans were compared by measuring absorbances with a spectrophotometer (UV-1601 PC; Shimadzu Co., Tokyo, Japan) at 510 nm.

***Reducing end levels of β-glucan*:** The levels of reducing end of β-glucans after γ-irradiation were determined by the 3,5-dinitrosalicylic acid (DNSA) method (19). Specifically, 1 mL of the irradiated sample was transferred into 15-mL glass tubes, and 2 mL of the modified DNSA reagent [0.5 g dinitrosalicylic acid, 8 g sodium hydrate, and 150 g Rochelle salt in 500 mL distilled water] was added to the tubes. The mixture was vortexed for 5 sec and boiled at 90°C for 10 min, and then cooled in ice. Absorbances of samples were measured at 550 nm by a spectrophotometer (UV-1601 PC; Shimadzu Co.), and the OD values were converted to levels of reducing ends of β-glucan using a standard curve prepared with different concentrations (0.625, 1.25, 2.5, 5, and 10 mg/mL) of glucose.

***Comparison of UV spectrum of β-glucans*:** Three mL of γ-irradiated and unirradiated β-glucan solutions (0.1 mg/mL) were transferred to quartz cuvettes and the UV spectra of the samples were then determined at a range of 200 and 500 nm by a spectrophotometer (UV-1610 PC; Shimadzu Co.).

Statistical analysis Means and standard deviations (SD) for results were calculated using SPSS software (Statistical Package for Social Sciences, 10.0, 2000). Differences among the mean values were examined by Student's two tails *t*-test (splenocyte proliferation and cytokine production) and Duncan's multiple comparison tests (levels of reducing end) with significant level of $p<0.05$.

Results and Discussion

Effect of irradiated β-glucans on splenocyte proliferation For the *in vitro* study, proliferation of splenocytes increased significantly in all β-glucan treated groups compared to the group treated with PBS ($p<0.05$), but no difference of

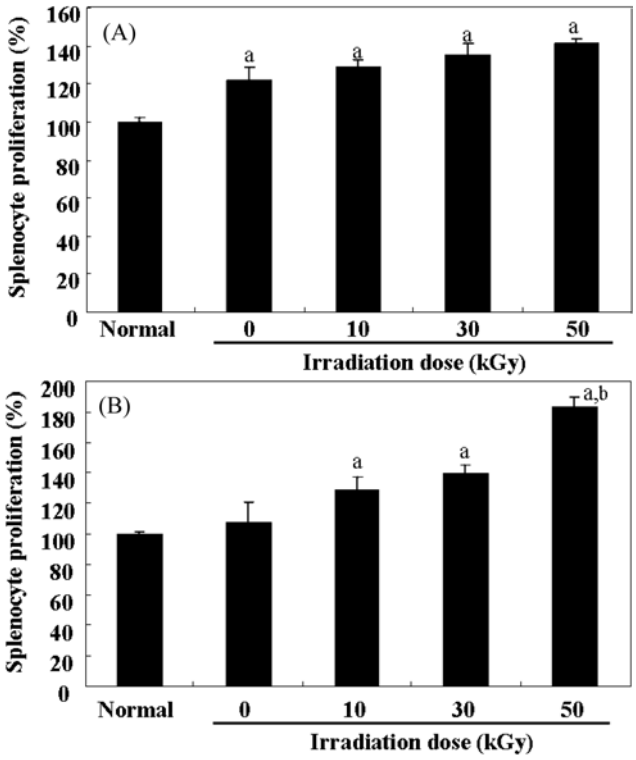

Fig. 1. Splenocyte proliferation in an *in vitro* (A) and an *in vivo* (B) model treated with unirradiated and irradiated β-glucans. [a]$p<0.05$ compared with PBS-treated group, [b]$p<0.05$ compared with unirradiated β-glucan (0 kGy)-treated group.

splenocyte proliferation was found between irradiated and unirradiated β-glucan (0 kGy) ($p\geq0.05$) (Fig. 1A). In the *in vivo* study, proliferation of splenocytes from the mice orally fed with β-glucan are shown in Fig. 1B. Unirradiated β-glucan (0 kGy) did not increase proliferation of splenocytes compared to the group fed with PBS ($p\geq0.05$). However, all irradiated β-glucans showed a significant increase in proliferation of splenocyte compared to the group fed with PBS ($p<0.05$). Unlike the results from the *in vitro* study, the proliferation of splenocytes from the mice fed with irradiated β-glucan at 50 kGy was higher than that of the mice fed with unirradiated β-glucan ($p<0.05$). This results may be caused by cell-to-cell interactions among various immune cells in *in vivo* system such as macrophage, dendritic cell, B cell, natural killer cell, mucosal immune cell. Therefore, these results showed that γ-irradiation improved β-glucan activity to increase splenocyte proliferation.

Effect of irradiated β-glucan on cytokine production

It has been reported that lymphocytes and monocytes isolated from mice treated with β-glucan produced a significant increase of cytokine which was generally characterized by enhancing IFN-γ production, suggesting that β-glucan may stimulate an immune response (20). In the *in vitro* study, the samples irradiated at 50 kGy were the only samples that increased the production of IFN-γ and IL-2 (Fig. 2A and 2C). For the *in vivo* study, all the β-glucan samples increased the production of IFN-γ and IL-2 compared to the group fed with PBS ($p<0.05$), and β-glucan irradiated at 50 kGy showed a greater IFN-γ and IL-2 production compared to unirradiated β-glucan ($p<0.05$). However, IFN-γ and IL-2 productions by the β-glucan samples irradiated at 10 and 30 kGy were not different with the cytokine productions by unirradiated β-glucan ($p\geq0.05$) (Fig. 2B and 2D). This result was also increase

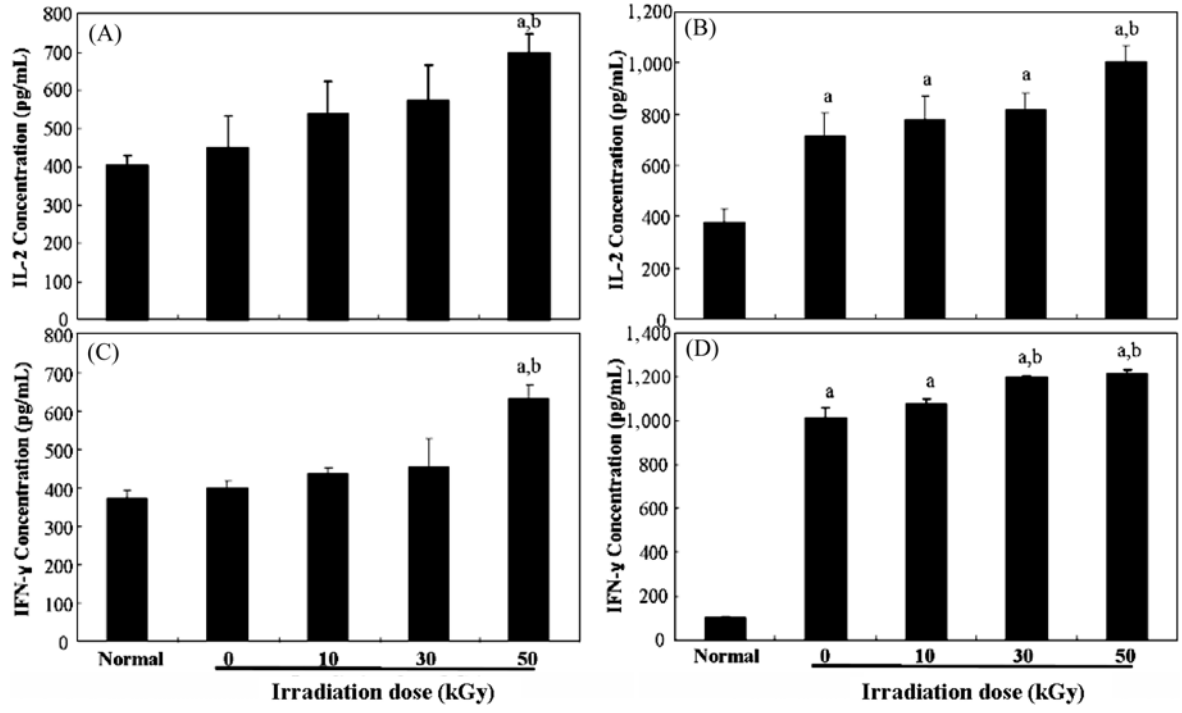

Fig. 2. Cytokine production in an *in vitro* (A, C) and an *in vivo* (B, D) model treated with unirradiated and irradiated β-glucans. [a]$p<0.05$ compared with PBS-treated, [b]$p<0.05$ compared with unirradiated β-glucan (0 kGy)-treated group.

Table 1. Concentrations (mg/mL) of β-1,3 and β-1,6 linkage (*n*=3) of β-glucan after γ-irradiation at various doses

Irradiation dose (kGy)	Concentration (mg/mL)	
	β-1,3 linkage	β-1,6 linkage
0	35.79[a1)]	11.01[a]
10	30.21[b]	9.50[ab]
30	25.96[c]	8.66[b]
50	24.88[c]	7.78[c]

[1)]Mean with the different superscripts in a same column are significantly different ($p<0.05$).

the cytokine productions in part of *in vivo* test. As discussed above, increase of immune response in *in vivo* may also be releated to cell-to-cell interaction among the immune cells in *in vivo* system.

Chen and Seviour (21) reported that there are correlations between immunological responses of β-glucan and its molecular structure, size, branching frequency, structural modification, conformation, and solubility. Moreover, Lehmann and Kunze (22) claimed in the patent that immunological responses by β-glucan were more effective at low molecular weight (1 to 30 kDa) than at high molecular weight (>100 kDa). A study by Byun *et al.* (17) showed that γ-irradition depolymerized β-glucan as the irradiation dose increased, which resulted in decreased molecular weight of β-glucan. Therefore, the results from our study may suggest that γ-irradiation produced a lower molecular weight of β-glucan, and this β-glucan improved immunological responses. In addition, this statement could be supported by the following results.

Effect of γ-irradiation on β-glucan depolymerization

Prior to irradiation, concentrations of the β-1,3 and β-1,6 linkages of β-glucan samples were 35.79 and 11.01 mg/mL, repectively (Table 1). However, the concentrations of both β-1,3 and β-1,6 linkages decreased to 24.88-30.21 mg/mL and 7.78-9.50% as the irradiation dose increased ($p<0.05$) (Table 1). To measure reducing ends of β-glucan produced from breakage of glycosidic bonds by irradiation, DNSA which binds to the reducing end of β-glucan was added to the irradiated β-glucan. The result (Fig. 3A) show that the reducing end levels of β-glucan increased as irradiation dose increased, suggesting that irradiation broke β-1,3 and β-1,6 linkages of β-glucan (Fig. 3A). Privious studies also showed that irradiation breaks the glycosidic linkages of polysaccharides, leading to the generation of a radical at the C_1 position on the glucose molecule or disaccharide in the presence of water (23).

Nagasawa *et al.* (24) demonstrated that a scission of the carbohydrates by γ-irradiation increased absorbances at 250 and 280 nm for carbonyl and carboxyl groups, respectively. Thus, in our study UV absorption of β-glucan before and after irradiation was compared at 250 and 280 nm. The result (Fig. 3B) shows that the UV absorption of β-glucan at 250 and 280 nm was increased by γ-irradiation. These results suggest that γ-irradiation broke the β-1,3 and β-1,6 linkages of β-glucan and lowered its molecular weight.

Acidic and enzymatic digestions are commonly used for the production of low molecular weight polysacharides, but

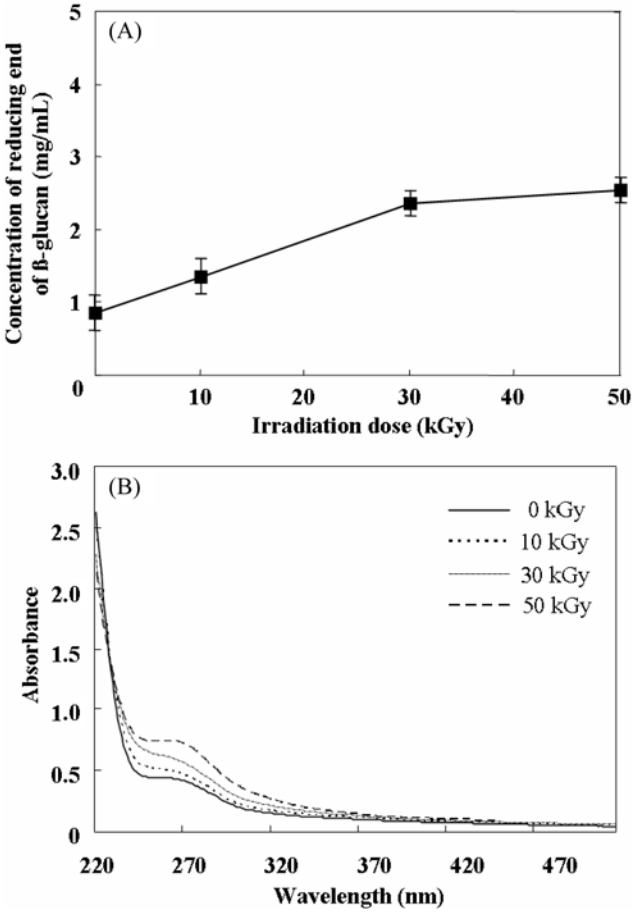

Fig. 3. Concentrations of reducing end (A) and UV spectra (B) of β-glucan after γ-irradiation at various doses.

they have high cost, low yield, long processing time, and waste (9), while γ-irradiation is simpler and more environmentally friendly than the chemical or enzymatic digestions. Hence, γ-irradiation could be an effective method to depolymerize high molecular weight β-glucan to low molecular weight β-glucan.

In conclusion, γ-irradiation could be an effective method to convert high molecular weight β-glucan to low molecular weight β-glucan. It was shown that the low molecular weight β-glucan increased the immunological activities of β-glucan.

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