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In Vitro Antioxidant Activity Profiles of β-Glucans Isolated from Yeast *Saccharomyces cerevisiae* and Mutant *Saccharomyces cerevisiae* IS2

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Abstract To explore the possible usefulness of β-glucans as natural antioxidants, the antioxidant profiles of β-glucan, extracted from *Saccharomyces cerevisiae* KCTC 7911, and water soluble and insoluble mutant β-glucan, isolated from yeast mutant *S. cerevisiae* IS2, were examined by five different *in vitro* evaluation methods: lipid peroxidation value (POV), nitric oxide (NO), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, reducing power, and β-carotene diffusion assay. The antioxidant activities of all β-glucans evaluated in POV test were comparable to or better than that of the known antioxidant, vitamin C. Remarkably, the β-glucan and water insoluble mutant β-glucan possessed 2.5-fold more potent activity than vitamin C at a dosage of 2 mg. Although vitamin C showed 100-fold greater activity than all β-glucans in NO and DPPH tests for measuring the radical scavenging capacity, all β-glucans revealed higher radical scavenging activity than the known radical scavenger, *N*-acetyl-L-cysteine (NAC), in DPPH test. The water insoluble mutant β-glucan had 2.6- and 5-fold greater antioxidative activity than water soluble β-glucan in NO and DPPH tests, respectively, showing that all β-glucans were able to scavenge radicals such as NO or DPPH. While all β-glucans revealed lower antioxidant profiles than vitamin C in both reducing power activity and β-carotene agar diffusion assay, the β-glucan and water insoluble mutant β-glucan did show a marginal reducing power activity as well as a considerable β-carotene agar diffusion activity. These results confirmed the potential usefulness of these β-glucans as natural antioxidants.

Keywords: β-glucan, water soluble and insoluble mutant β-glucan, antioxidant activity, vitamin C, *N*-acetyl-L-cysteine

Introduction

The β-glucan [(1,3)-β-D-glucan], carbohydrate polymer extracted from the inner cell wall of the yeast *Saccharomyces cerevisiae* has been shown to possess various biological activities such as immunostimulation, growth inhibition of tumor cells, enhancement of infection resistance, and improvement of wound healing (1-5). In addition, yeast β-glucan showed a lowering effect for plasma low-density lipoprotein (LDL) concentrations, while increasing high-density lipoprotein (HDL) cholesterol in hypercholesterolemic obese men (6). Recently, the mutant β-glucan, isolated from the cell wall of yeast mutant *S. cerevisiae* IS2, has been examined for its immunomodulating activity, showing that the mutant β-glucan possessed greater potential immuno-stimulating activity than that of wild-type, *S. cerevisiae* KCTC 7911 (7). This mutant β-glucan was extracted from the cell wall of yeast mutant *S. cerevisiae* IS2, obtained by the treatment of *S. cerevisiae* KCTC 7911 with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG) (8). The mutant β-glucans were highly resistant to zymolyase activity, responsible for the degradation of β-(1,3)-D-glucopyranosyl side chains of β-glucan and the mutant β-glucan particles were also stable under mechanical disruption with glass beads.

In an effort to discover an inherent but unknown biological activity elicited by β-glucans, we first evaluated the antioxidant activity of β-glucan (wild type) and mutant β-glucans, i.e., water soluble- and insoluble mutant β-glucan, since little research attention has focused on the antioxidant effect induced by these glucans.

To explore the possible usefulness of all β-glucans as natural antioxidants, *in vitro* antioxidant activities induced by glucans were measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, nitric oxide (NO), lipid peroxidation value (POV), reducing power, and β-carotene agar diffusion assay. In addition, their antioxidant activities were compared with two well known antioxidants: vitamin C and *N*-acetyl-L-cysteine (NAC) (9-12).

We now report the *in vitro* antioxidant activity profiles induced by β-glucan and water soluble and insoluble mutant β-glucan, and postulate the usefulness of these β-glucans as possible natural antioxidants.

Materials and Methods

Spectrophotometric analysis of antioxidant activity was performed on a DR/4000U UV-Vis spectrophotometer (HACH Co., Loveland, CO, USA). Griess reagent was prepared from the 1:1 mixture of solution A, containing 2 %(w/v) sulfanilamide and 4%(w/v) phosphoric acid, and solution B, containing 0.2%(w/v) naphthyl-ethyl-enediamide. Other chemicals and solvents were from Aldrich Chemical Company (Milwaukee, WI, USA).

Preparation of β-glucan The β-glucan was isolated from *S. cerevisiae* KCTC 7911 as described previously (13).

Preparation of mutant β-glucans The water soluble and insoluble mutant β-glucans were prepared from yeast cell wall mutant *S. cerevisiae* IS2 as previously described,

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with slight modifications (7, 13, 14), and the preparation was carried out by Research Institute, Natural F&P Co., Ltd (Seoul, Korea). Briefly, *S. cerevisiae* IS2 (80 g) was added to 3% NaOH (1,000 mL) and heated to 90-95°C for 60 min with vigorous stirring. Following separation by centrifugation (800×g, 15 min), the lysed yeast cells were resuspended in 3% NaOH (2,000 mL), boiled to 75°C for 180 min and allowed to stand at room temperature for 24 hr. Following centrifugation (800×g, 15 min), the lysed yeast cells were resuspended in fresh water (2,000 mL), with the pH adjusted to 4.5 by HCl addition. The suspension was then stirred at 75°C for 60 min and centrifuged at 800×g for 15 min to obtain the precipitate, which washed three times with distilled water and twice with ethanol, and then dried as particulate glucan in a drying oven (water insoluble mutant β-glucan). The aqueous glucan suspension was collected by standard methods to give water soluble, mutant β-glucan.

Nitric oxide (NO) radical scavenging activity The NO radical scavenging activities from all β-glucans, NAC and vitamin C were essentially determined as described previously (15, 16). Each reaction mixture, containing 0.5 mL of the various diluted test compounds with 0.02 M phosphate buffer (pH 7.4) and 0.5 mL of fresh sodium nitroprusside solution, formed by dissolving 0.01 mM sodium nitroprusside in 0.02 M phosphate buffer (pH 7.4) was shaken and incubated for 150 min in a water bath at 25°C. Finally, 1 mL of Griess reagent was added to the incubated sample and the scavenging activity of the samples was measured by the absorbance at 542 nm.

DPPH radical scavenging activity The antioxidant activities of all β-glucans, NAC, and vitamin C were determined by bleaching the stable DPPH (17, 18). The reaction mixture contained 2 mL of ethanol, 0.5 mL (100 μM) of DPPH in ethanol, and 1 mL of test compounds in distilled water in a total volume of 3.5 mL. Each testing solution was diluted to four or five concentrations in series. The reaction mixture was allowed to stand for 10 min at room temperature and the intensity of red-purple chromophore measured at 517 nm.

Lipid peroxidation value (POV) POVs from all β-glucans, NAC, and vitamin C were determined as previously described with slight modifications (19-21). Water soluble mutant β-glucan (5, 15 mg) and water insoluble mutant β-glucan (1, 2 mg) were dissolved in 50 % ethanol (1 mL). A screw-capped, 50 mL, round-bottomed test tube containing linoleic acid (0.13 mL), 0.05 M sodium phosphate buffer (10 mL, pH 7.4), pure ethanol (9.5 mL), and either test compounds, mutant β-glucans (1 mL) or vitamin C (10 mM), adjusted with distilled water to a final volume of 25 mL, was shaken and incubated in a water bath at 40°C. Blank controls contained 50% ethanol instead of compounds. To the 1 mL incubated sample was added in succession 4.7 mL of 75% ethanol, 0.1 mL of 30% NH_4SCN, and 0.1 mL of 0.02 M $FeCl_2$ in 3.5% HCl. The sample tube was thoroughly vortexed and allowed to stand for 3 min. POV was estimated by absorbance at 500 nm. Calculations of their activities were modified. The straight-line equations of peroxide values in the samples and control were primarily determined by regression analysis for calculation of relative lipid POV rate. The peroxide values were measured at intervals during an incubation period of 20 days. The relative lipid POV rates were calculated with the slopes of the straight-line equations. The calculation formula of relative antioxidant activity is as follows:

$$\text{Relative antioxidant activity (\%)} = (1 - As/Ac) \times 100$$

where, As is the slope of the straight line equation of the sample and Ac is the slope of the straight line equation of the control.

Reducing power assay Relative reducing powers of all β-glucans, NAC, and vitamin C were determined as previously described with slight modifications (22). Each compound in 1 mL of distilled water was mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%, w/v) and then the mixture was incubated at 50°C for 30 min. Trichloroacetic acid (10 %, w/v, 2.5 mL) was added to the mixture, which was centrifuged at 900×g for 10 min. Finally, 1 mL of the upper layer solution was mixed with 1 mL of distilled water and 0.2 mL of $FeCl_3$ (0.1%, w/v), and the absorbance was estimated at 700 nm. Increased absorbance of the reaction mixture indicated stronger reducing power.

β-Carotene agar diffusion assay β-Carotene agar diffusions of all β-glucans, NAC, and vitamin C were carried out as previously described with slight modifications (23). Agar solution (2%, 100 mL) containing β-carotene (1 mg) in 10 mL of acetone and linoleic acid (4 mg) in 2 mL of ethanol was distributed to a polystyrene tissue culture petri dish (10 mL, 100 mm, Costar, Cambridge, MA, USA) and allowed to solidify at room temperature. Each various diluted test compound was applied with distilled water (0.1 mL) to the hole (40 mm diameter) in agarose gel and incubated overnight at 45°C. The diameters of the yellow circle formed from each sample were measured.

Results and Discussion

The roles of β-glucans as biological response modifiers and potential immunotherapeutics have been described. Based on the various therapeutic potentials of glucans, we undertook studies to develop β-glucans as natural antioxidants. Therefore, the primary objective of this study was to examine the inherent antioxidant status of β-glucan, extracted from *S. cerevisiae* KCTC 7911, as well as that of water soluble and insoluble mutant β-glucan, isolated from yeast mutant *S. cerevisiae* IS2, in order to explore the potential as natural antioxidants.

The individual antioxidant activities of β-glucan, and water soluble and insoluble mutant β-glucan were measured and compared with those of two known antioxidants, vitamin C and NAC, in five different *in vitro* evaluation methods: DPPH, NO, lipid POV, reducing power, and β-carotene diffusion assay.

The antioxidant activity profiles of all β-glucans evaluated in POV test were comparable to or better than

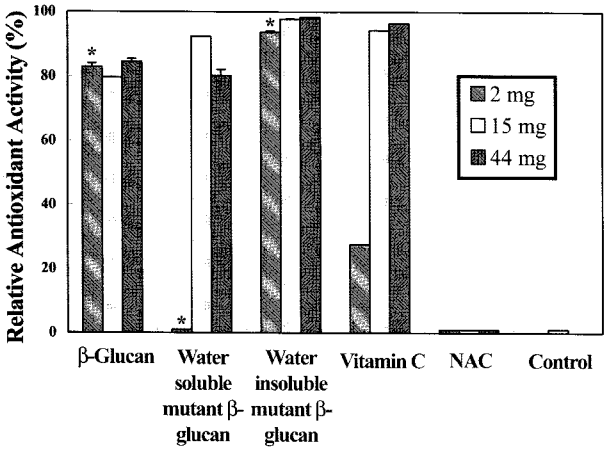

Fig. 1. Relative antioxidant activities by β-glucan, mutant β-glucans, and vitamin C in POV tests. Data are presented as mean±SD. For analysis of the two groups, the unpaired Students *t*-test was performed. A significant difference in POV between glucans (2 mg) and vitamin C is indicated by $*p<0.05$.

Table 1. Radical scavenging capacities of β-glucans, vitamin C, and *N*-acetyl-L-cysteine (NAC) in nitric oxide (NO) and DPPH tests

Compounds	SC_{50} in NO	SC_{50} in DPPH
β-Glucan (mg)	4.52±0.4	11.03±0.42
Water soluble mutant β-glucan (mg)	19.7±2.8	41.1±12.6
Water insoluble mutant β-glucan (mg)	7.6±1.8	8.1±0.8
Vitamin C (mg)	0.08±0.01	0.02±0.00
N-acetyl-L-cysteine (mg)	0.04±0.00	50±0.00

The correlation coefficients between the straight line equations and NO and DPPH test values were all above 0.9 ($r \geq 0.9$). Data are mean ± SD of three to five experiments.

that of the known antioxidant, vitamin C (Fig. 1). Especially, β-glucan and water insoluble mutant β-glucan showed much higher relative antioxidant activity than that of vitamin C at 2 mg of dosage, indicating that these β-glucans possessed a potential lipid POV and demonstrated promise as natural antioxidants. Interestingly, NAC was devoid of antioxidant activity in POV test (Fig. 1).

In order to investigate the possible radical scavenging capacity elicited by all β-glucans, we then examined the ability of these β-glucans to scavenge radicals, i.e., NO and DPPH, *in vitro* and compared the ability with that of two known radical scavengers: vitamin C and NAC (8-11). Although vitamin C showed 100-fold greater antioxidant activities than all β-glucans in both NO and DPPH tests for measuring the radical scavenging capacity, it is worthwhile to note that all β-glucans revealed higher radical scavenging activity than the known radical scavenger, NAC, in DPPH test (Table 1). All glucans exhibited various degrees of radical scavenging capacity with β-glucan being the most potent in NO test and water insoluble mutant β-glucan in DPPH test, showing that all β-glucans were able to scavenge the NO and DPPH radicals. The water insoluble mutant β-glucan was 2.6- and 5-fold more antioxidative than water soluble mutant β-glucan in NO and DPPH tests, respectively (Table 1). Thereby, these β-glucans might serve as naturally occurring antioxidants with radical scavenging activity.

In the reducing power test, although the degree of reducing power of β-glucans showed no distinguishable concentration-dependent change at concentrations of 10-500 mg, all glucans revealed a marginal reducing power activity (Table 2). However, their reducing activities were lower than that of vitamin C or NAC (Table 2).

The abilities of β-carotene diffusion by β-glucan and mutant β-glucans were measured and also compared with two known antioxidants: vitamin C and NAC. In this assay system, β-glucan and water insoluble mutant β-glucan showed increased antioxidant activities in a dose-dependent manner (10, 15, 20 mg), although their activities revealed lower antioxidant profiles than the known antioxidant, vitamin C (Fig. 2). The antioxidant activities of NAC were not especially increased at all compared to those of the controls in β-carotene diffusion assay (Fig. 2), demonstrating that these β-glucans have the ability of β-carotene diffusion antioxidant profiles.

Synthetic antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and propyl gallate (PG) are used in various foods to delay or prevent the onset of oxidative rancidity (24). However, there is serious concern about the toxic and carcinogenic effects of these compounds. Therefore, much research attention has focused on pursuing new natural antioxidants and on substituting synthetic antioxidants with natural antioxidants (25, 26).

In this *in vitro* antioxidant evaluation study, all β-glucans revealed potential antioxidant profiles in POV test and proved to have both effective radical scavenging activities and marginal reducing powers. The antioxidative

Table 2. Comparison of reducing powers of β-glucan, mutant β-glucans, vitamin C, and *N*-acetyl-L-cysteine (NAC)

Compounds	Absorbance at 700 nm			
	10 μg	50 μg	100 μg	500 μg
β-Glucan	0.262±0.001	0.263±0.001	0.265±0.005	0.268±0.003
Water soluble mutant β-glucan	0.257±0.001	0.262±0.006	0.263±0.006	0.268±0.010
Water insoluble mutant β-glucan	0.263±0.003	0.263±0.003	0.265±0.003	0.287±0.015
Vitamin C	0.368±0.025	0.715±0.028	1.113±0.054	3.422±0.011
N-acetyl-L-cysteine	0.301±0.019	0.405±0.008	0.516±0.012	1.241±0.077

The values represent the mean±SD of three experiments. Reducing power of control was 0.253±0.03 at absorbance of 700 nm.

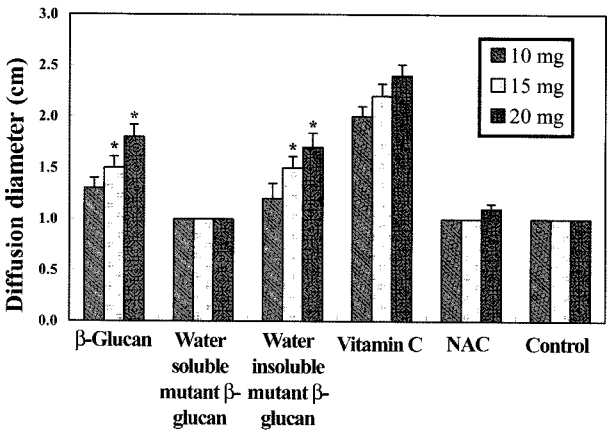

Fig. 2. β-Carotene diffusions by β-glucan, mutant β-glucans, *N*-acetyl-L-cysteine (NAC), and vitamin C. Data are summarized as mean±SD. For analysis of the two groups, the unpaired Students *t*-test was performed. A significant difference in β-carotene diffusion between glucans (10 and 15 mg) and NAC or control is indicated by $*p<0.05$.

activities of β-glucan and water insoluble mutant β-glucan toward β-carotene diffusion were active and comparable to those of vitamin C. These results indicate that all β-glucans show promise as potential natural antioxidants. Especially, water insoluble mutant β-glucan possesses a meaningful antioxidant profile *in vitro*, even though the mechanism(s) for this behavior remains to be elucidated. We therefore suggest that further studies exploring the clinical usefulness of these β-glucans are conducted to evaluate whether β-glucans can be useful natural antioxidants in conjunction with immunotherapeutics.

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