J. Microbiol. Biotechnol. (2006), **16**(4), 576–583

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Immune-Enhancing Alkali-Soluble Glucans Produced by Wild-Type and Mutant *Saccharomyces cerevisiae*

HA, CHANG HOON[1], KI HONG LIM[1], SE HWAN JANG[1], CHEOL WON YUN[1], HYUN DONG PAIK[2], SEUNG WOOK KIM[3], CHANG WON KANG[2], AND HYO IHL CHANG[1]*

[1]*School of Life Science and Biotechnology, Korea University, Seoul 136-701, Korea*
[2]*Division of Animal Life Science, Kon-Kuk University, Seoul 143-701, Korea*
[3]*Department of Chemical and Biological Engineering, Korea University, Seoul, 136-701, Korea*

Received: August 16, 2005
Accepted: November 17, 2005

Abstract The alkali-soluble glucan of the yeast cell wall contains β-(1,3)- and (1,6)-D-linkages and is known to systemically enhance the immune system. In the previous study [6], in order to isolate cell wall mutants, a wild-type strain was mutagenized by exposure to ultraviolet light, and the mutants were then selected via treatment with laminarinase (endo-β-(1,3)-D-glucanase). The mass of alkali- and water-soluble glucans produced by the mutant was measured to be 33.8 mg/g of the dry mass of the yeast cell. Our results showed that the mutants generated the amount of alkali-soluble glucan 10-fold higher than that generated by the wild-type. Structural analysis showed that the alkali-soluble glucan from the mutants was associated with a higher degree of β-(1,6)-D-linkage than was observed in conjunction with the wild-type. Yeast cell wall β-glucan was shown to interact with macrophages via receptors, thereby inducing the release of tumor necrosis factor alpha (TNF-α) and nitric oxide. Alkali-soluble β-glucans, both from water-soluble and water-insoluble glucan, exhibited a higher degree of macrophage activity with regard to both the secretion of tumor necrosis factor alpha (TNF-α) and nitric oxide and direct phagocytosis, than did the positive control (1 μg of lipopolysaccharide).

Key words: Alkali-soluble glucan, mannoprotein, yeast cell wall, random mutation

The cell wall of *Saccharomyces cerevisiae* is composed of three layers that are partially merged together: an inner layer of alkali-insoluble β-glucan, a middle layer of alkali-soluble β-glucan, and an outer layer of glycoprotein, which contains carbohydrates in the form of phosphorylated mannan [21]. The cell wall harbors β-(1,3)-glucan, β-(1,6)-glucan, chitin, and several mannoproteins. The polysaccharides appear to perform structural functions, whereas the mannoproteins may act as "filler" and perform important functions involving the permeability of the cell wall. All four of the structural components of the cell wall, β-(1,3)-glucan, β-(1,6)-glucan, chitin, and mannoprotein, are linked. The proportions of these components tend to vary among species, but approximately equal proportions of mannan and glucan have been found in *S. cerevisiae*, and the amounts of alkali-soluble glucan and alkali-insoluble glucan also tend to be roughly equal [5].

β-Glucans are structurally complex glucose homopolymers, which are normally isolated from yeast or fungi. The number of individual glucans that have been determined to exist is almost as great as the number of sources from which it has been isolated. Physicochemical parameters, including solubility, primary structure, molecular weight, branching, and polymer charge, have been shown to exert some influence over the biological activities in which the β-glucans are engaged. Yeast cell wall β-glucan is a glucose homopolymer that is linked either by β-(1,3)- or β-(1,6)-D-glycosidic bonds, and it is the most abundant polysaccharide in the yeast cell wall and represents approximately 12–14% of the dry weight of the cell. The glucans are grouped according to the degree to which they are soluble in alkali. The alkali-soluble glucans constitute a minor component (15–20% w/w of total glucan) of the cell wall, and have little structural importance. The major glucan component in the cell wall is in fact the alkali-insoluble variety. This glucan consists of a β-(1,3)-linked backbone with a high molecular weight (240,000), which contains 3% β-(1,6)-interaction linkages, and is responsible for both the structure and integrity of the cell walls [10]. The glucan is generally separated into two classes: the β-(1,3)-glucans and the β-(1,6)-glucans. The β-(1,3)-glucans consist primarily of the linear β-(1,3)-linked glucans, although there is also

*Corresponding author
Phone: 82-2-3290-3421; Fax: 82-2-923-9923;
E-mail: hichang@korea.ac.kr

some branching via β-(1,6) linkage. This class of glucan has an average of 1,500 glucose residues per molecule, and tends to form insoluble fibers. The β-(1,6)-glucan predominantly harbors β-(1,6) linkages, but also contains some β-(1,3)-linked branch points and exhibits a degree of polymerization of approximately 140 glucose residues. The β-(1,6)-glucan exhibits some β-(1,3)-linked branches, and it is to these branches that the reducing termini of the chitin chains appear to be attached via β-(1,4) or β-(1,2)-linkages. Finally, the reducing terminus of the β-(1,6)-glucan has been shown to connect to the nonreducing terminal glucose of β-(1,3)-glucan, although the linkage by which this is accomplished remains to be established. The linkage between the mannoproteins and β-(1,6)-glucan plays a central role in the organization of the cell wall of yeast. In this complex, the chitin is attached directly to a branch of β-(1,6)-glucan [13, 11]. The β-(1,3)-glucan and the β-(1,6)-glucan cross-link to each other, and it has been suggested that β-(1,6)-glucan might be associated with chitin.

Polysaccharides, especially glucans, have long been known as immunomodulators. Interest in glucans became far more intense, when it was demonstrated that zymosan can stimulate macrophages via the activation of the complement system [18]. Alkali-soluble β-glucan functions as a nonspecific immune system modulator. The biological activities of alkali-soluble glucans include host-mediated antitumor activity, adjuvant effects, and the activation of neutrophils, eosinophils, macrophages, and complements [7, 12, 22, 24–27, 35]. Increasing evidence suggests that β-(1,3)-glucans can induce nonspecific inflammatory reactions. Therefore, particularly in recent years, increased attention has been focused on the cell wall components that have been administered as immunoadjuvants, antitumor, and radioprotective agents via immune system stimulation. However, a problem lies in the fact that water-insoluble or barely soluble β-glucans can induce significant adverse effects (granuloma formation, inflammation, microembolization, pain), when parenterally administered [4, 15, 16, 34]. The water-solubility of β-glucan appears to be, at least in part, dependent on the intensity of the β-(1,6)-D-glycosidic bonds [1].

We earlier prepared a mutant of the cell wall of *Saccharomyces cerevisiae*. In the present study, we attempted to characterize the capacity of β-glucans to stimulate the human immune system by quantifying the degree to which phagocytosis occurred and by measuring levels of both TNF-α and nitric oxide.

MATERIALS AND METHODS

Strains and Cultivation

Saccharomyces cerevisiae JH (Hansen1883, Mat a/Mat α) was used as a wild-type strain. *S. cerevisiae* JUL3 was isolated from JH (Fig. 1). All of the *S. cerevisiae* strains

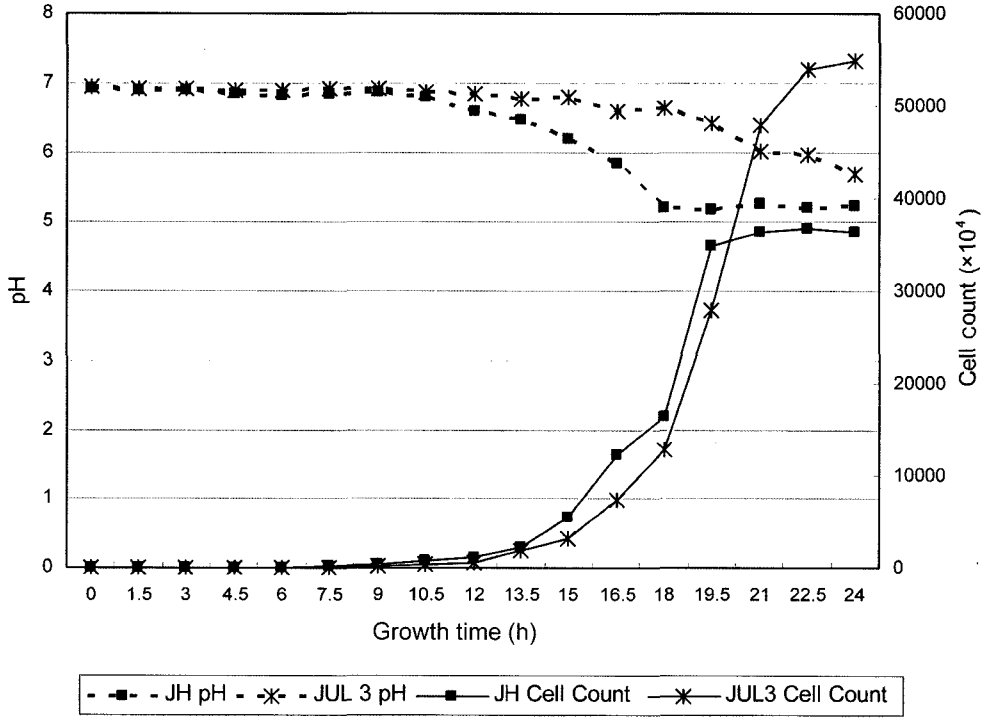


Fig. 1. Cultivation curve of yeast, JH (wild-type) and JUL3 (mutant).
The growth of the cells was measured by changes of cell number and pH of media. JUL3 (mutant strain) exhibited lower growth rate than JH (wild-type).

tested were allowed to grow in complex YPD [yeast extract 2%, bactopeptone 4%, glucose 4%] at 30°C.

Extraction of Alkali-Soluble Glucans

Alkali-soluble β-glucans were extracted and purified by the combination of the extraction methods for β-(1,3)- and β-(1,6)-glucans, as described in the previous study [6].

Measurement of Molecular Weights of Alkali-Soluble Glucans

The molecular weights of the β-glucans were measured by a high-performance size-exclusion chromatography attached with multiangle laser light scattering and refractive index detectors (HPSEC-MALLS-RI). The system was composed of an SEC column (TSK gel 3000PW, Tosoh, Japan), a multiangle laser light scattering detector (Dawn DSP-F, Wyatt Technology, U.S.A.), and RI detector (Shodex SE71, Tokyo, Japan). Output voltages at the 18 angles were used to calculate the molecular weight of the samples. A mobile phase of 0.05 M NaOH was passed through cellulose acetate filter (0.2 μm pore size) prior to use. The flow rate was 0.5 ml/min.

Cell Culture

A murine macrophage cell line, RAW 264.7, and a murine fibroblast cell line, L929, were acquired from the Korea Cell Line Bank (KCLB, Seoul, Korea) (Table 1). The cells were grown in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Grand Island, U.S.A), 100 U/ml of penicillin, and 100 μg/ml streptomycin.

Bioassay of Nitric Oxide

The 24-h macrophage supernatants (100 μl) were assayed for nitric oxide via the Griess reaction, in accordance with a described microassay method [3]. In brief, Griess reagent (1% sulfanilamide/0.1% naphthylenediamine dihydrochloride/ 2.5% H_3PO_4) was incubated with an equal volume of macrophage supernatants, which had been treated with β-glucan, for 10 min at room temperature, and absorbance was measured at 550 nm with a micro-ELISA reader. Nitric oxide concentrations (nmol/well) were then determined, using $NaNO_2$ as a standard.

L929 Cytotoxicity Assay

The levels of TNF-α in the medium were assessed by a modified L929 cytotoxicity assay [8, 9, 14, 17]. Murine L929 monolayers were grown to confluence in RPMI 1640 medium containing 10% FBS, harvested with trypsin, and then plated on 96-well plates overnight at 37°C. The next day, the medium was exchanged with RPMI 1640 medium containing actinomycin D (1 μg/ml). Recombinant murine TNF-α (Sigma) was employed as a relative standard of bioactivity in these assays. Standards or samples were then plated onto the L929 cells, and the cells were incubated for 18 h at 37°C. Thereafter, the medium was removed, and the cells were stained with 0.5% crystal violet, after which the optical density was determined at 490 nm. A standard concentration curve was prepared using the optical densities of the standards, and this was also employed in the calculation of the levels of TNF-α in the samples.

Phagocytosis Assay

Phagocytic activity was assayed according to the previously described method [24]. In brief, Raw 264.7 cells (1×10^5 cells/well) washed twice with 100 μl of RPMI 1640, were incubated with 1×10^7 of FITC-labeled *E. coli* (4×10^6 cells/well) (Molecular Probes, Eugene, U.S.A.) in RPMI 1640 containing 10% FBS in 96-well flat-bottomed tissue culture plates for 30 min at 37°C. The macrophages were then washed 3 times with warmed PBS, and were solubilized by the addition of 100 μl of 50 mM sodium cholate. The intensity of fluorescence was determined with a microplate reader at an excitation wavelength of 490 nm and an emission wavelength of 530 nm.

Results and Discussion

Purification and Determination of the Cell Wall Glucans

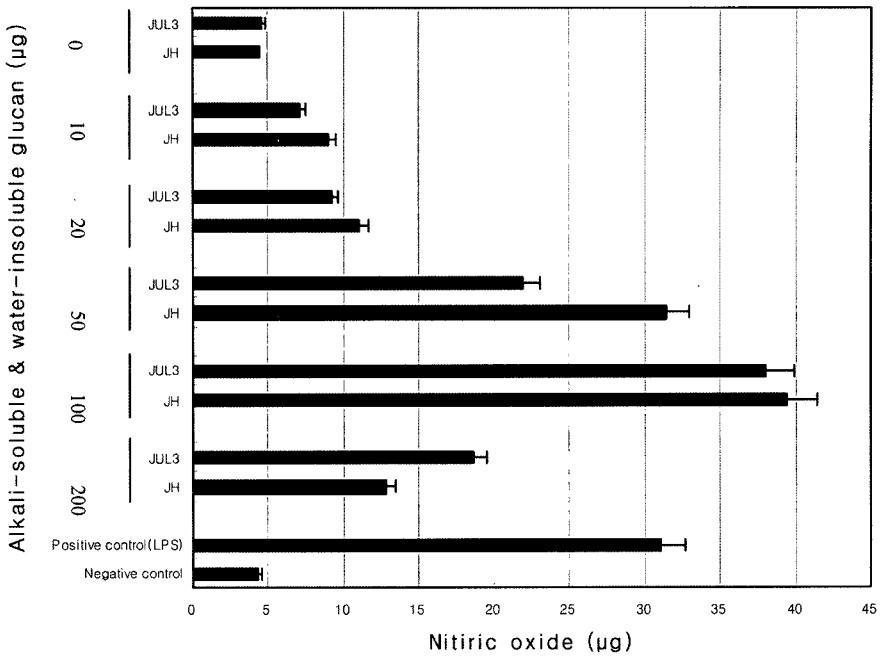
In the previous study [6], in order to isolate mutants with high β-glucan yields, we mutated the wild-type JH strain

Table 1. Yield and Molecular weights of β-glucan fractions

	JH (Wild-type)		JUL3 (Mutant)	
	Alkali-soluble and water-insoluble β-glucan	Alkali-soluble and water-soluble β-glucan	Alkali-soluble and water-insoluble β-glucan	Alkali-soluble and water-soluble β-glucan
Yield (mg/g of cell dry mass)	80.5	3.6	141.6	31.8
Molecular weight (×10,000)	52	118	60	172

The molecular weight of these glucans was measured by HPSEC-MALLS-RI. The yield of the alkali-soluble glucan in the mutants was ten-fold higher than that in the wild-type. The ratio of molecular weights of the water-soluble β-glucan was 1:1.46 (JH:JUL3). The results represent the mean of two independent experiments±SD.

A. Alkali-soluble and water-insoluble β-glucan



B. Alkali-soluble and water-soluble β-glucan

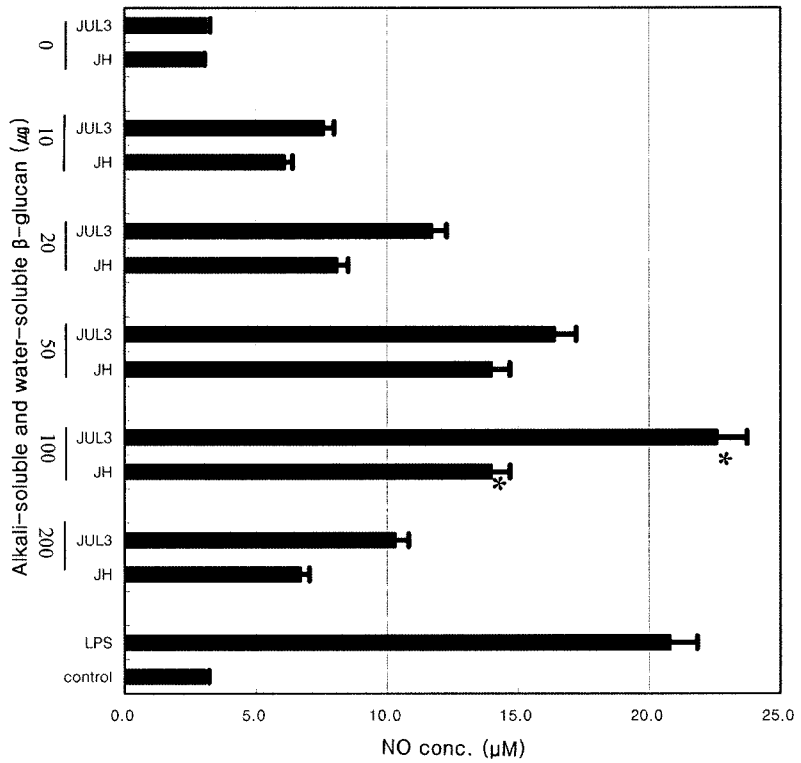


Fig. 2. Effect of gradient injection of *in vitro* β-glucans stimulation on NO production.
RAW 264.7 cells (Murine Mφ, 1×10^6 cells/each well) pretreated with different doses (0, 10, 20, 50, 100, 200 μg) of β-glucan were incubated for 21 h. NO production was measured with a microplate spectrophotometer at 520 nm. LPS (1 μg/ml) was used as a positive control. The results represent the mean of four independent experiments±SD. *Represents significant difference between wild-type (JH) and mutant (JUL3) at $P<0.05$ level.
A. Standard equation – NO (μM)=(Y(OD)-0.035)/0.0011.
B. Standard equation – NO (μM)=(Y(OD)-0.04)/0.009.

via UV irradiation, and then selected the glucanase-resistant strains by screening for laminarinase [endo-β-(1,3)-glucanase] resistance.

The mutant strain, JUL3, exhibited a lower growth rate than wild-type (Fig. 1). The cell wall mutant generated the amount of alkali-soluble glucan 10-fold of that of the wild-type, and the alkali-soluble and water-soluble β-glucan was found to have a much higher molecular weight than the alkali-soluble and water-insoluble β-glucan (Table 1). In particular, the molecular weights of the water-soluble and water-insoluble glucans in the mutant differed substantially: The molecular weights of the water-soluble glucan in the mutant were measured to be 1.72×10^6 (JUL3). These values were about three times of that determined for the water-insoluble glucans. Our results indicate that the marked increment of alkali- and water-soluble glucans from the mutants might be attributable to increased polymerization of the β-(1,6)-linked branch in the β-(1,3)-glucans.

Therefore, we evaluated the degree to which immunoactivity was enhanced in the alkali-soluble and water-soluble β-glucan. The effects of various glucans on macrophages have been fairly well established. However, in order to show that the alkali-soluble and water-soluble glucan of the cell wall mutants truly possessed an immune-enhancing characteristic, an evaluation of nitric oxide and TNF-α production, in macrophages, is required.

Analysis of Immune Activity of Cell Wall Glucans

We measured the effects of different doses of each type of glucans on nitric oxide and TNF-α production in the macrophages (Raw 264.7). The generation of nitric oxide represents an important component of the host's immune response against foreign infection. As indicated in Fig. 2, the macrophages can be compelled to release nitric oxide when incubated with β-glucans. The alkali-soluble and water-soluble glucan was determined to be about 0.75-fold less effective in the induction of nitric oxide generation than the alkali-soluble and water insoluble glucan; however, it exhibited an ability to enhance immune activity comparable with that of the positive control, LPS (lipopolysaccharide). Higher concentrations of the alkali-soluble and water-insoluble glucan as well as the alkali-soluble and water-soluble glucan (over 200 μg) were consistently found to induce an apparent suppression of nitric oxide release from the RAW 264.7 cells.

The results summarized in Fig. 3 indicate that the effects of the alkali-soluble and water-soluble β-glucans on TNF-α generation in Raw 264.7 cells were much less profound than the same effects of the alkali-soluble and water-insoluble β-glucan. Maximal TNF-α release was observed when higher concentrations of β-glucans were used (10 to 1,000 μg/ml). TNF-α from Raw 264.7 cells was released less abundantly by the cell wall mutant, JUL3, than by the wild-type, JH, at a dose of 1,000 μg. However, a different situation was observed in the case of TNF-α, in which the alkali-soluble and water-soluble β-glucan of the wild-type cell manifested higher levels of TNF-α release than did the glucan of the cell wall mutants. However, TNF-α levels induced by any of the glucans tested were consistently significantly more abundant than was observed with the negative control.

Phagocytosis has long been recognized as one of the principal functions of macrophages, and its role in the process of macrophage differentiation has been extensively studied [28–30]. Therefore, we attempted to assess β-glucan ability to modulate antibacterial activity in the macrophages (phagocytosis). The macrophages were tested for their ability to phagocytize FITC-labeled *E. coli* bacteria, and experimental data are shown in Fig. 4. At 10:1 bacteria/RAW 264.7 (murine macrophage) ratio, the β-glucans-treated cells exhibited more profound antibacterial activity, evidenced by both the percentage of cells engaged in phagocytosis and the mean number of bacteria phagocytized per cell. These results were reproducible in several independent experiments. The number of cells substantially increased 60 min after treatment, and these elevated levels persisted as long as 120 min. Compared with the negative control (no glucan-treatment Raw 264.7 cell line), all of the β-glucans variants exhibited higher levels of macrophage antibacterial activity than did the negative control. Although the water-soluble glucan manifested a low level of phagocytic activity, approximately 5,000 RFU (relative fluorescence unit) at 120 min, this level of activity was still substantially higher than that of the negative control. In particular, with regard to the water-soluble glucan, the mutants exhibited significant difference in phagocytic activity, compared with that of the wild-type. However, there was no difference in the water-insoluble glucans between the wild-type (JH) and the cell wall mutants (JUL3).

Macrophages have also been established to perform a central function in the generation of specific and nonspecific immunity. The alkali-soluble glucans have been demonstrated to stimulate TNF-α under both *in vivo* and *in vitro* conditions [23, 32]. TNF-α generation results in the exertion of a protective effect against infections [19]. It has been hypothesized that glucans exert an enhancing effect on leukocyte functions via increased TNF-α secretion, particularly during the earlier stages of infection. A certain glucan receptor protein for alkali-soluble glucan has been found to exist on the surfaces of the macrophages [2]. Therefore, alkali-soluble glucan appears to clearly interact with specific receptors that are present on the mononuclear phagocytes, and stimulates nitric oxide generation. Alkali-soluble glucan has been shown to mediate the enhancement of nitric oxide synthesis by macrophages, and this has been attributed to direct activation of macrophages by alkali-soluble glucans [20]. Because of the huge difference in the activities of various glucans that have been isolated from

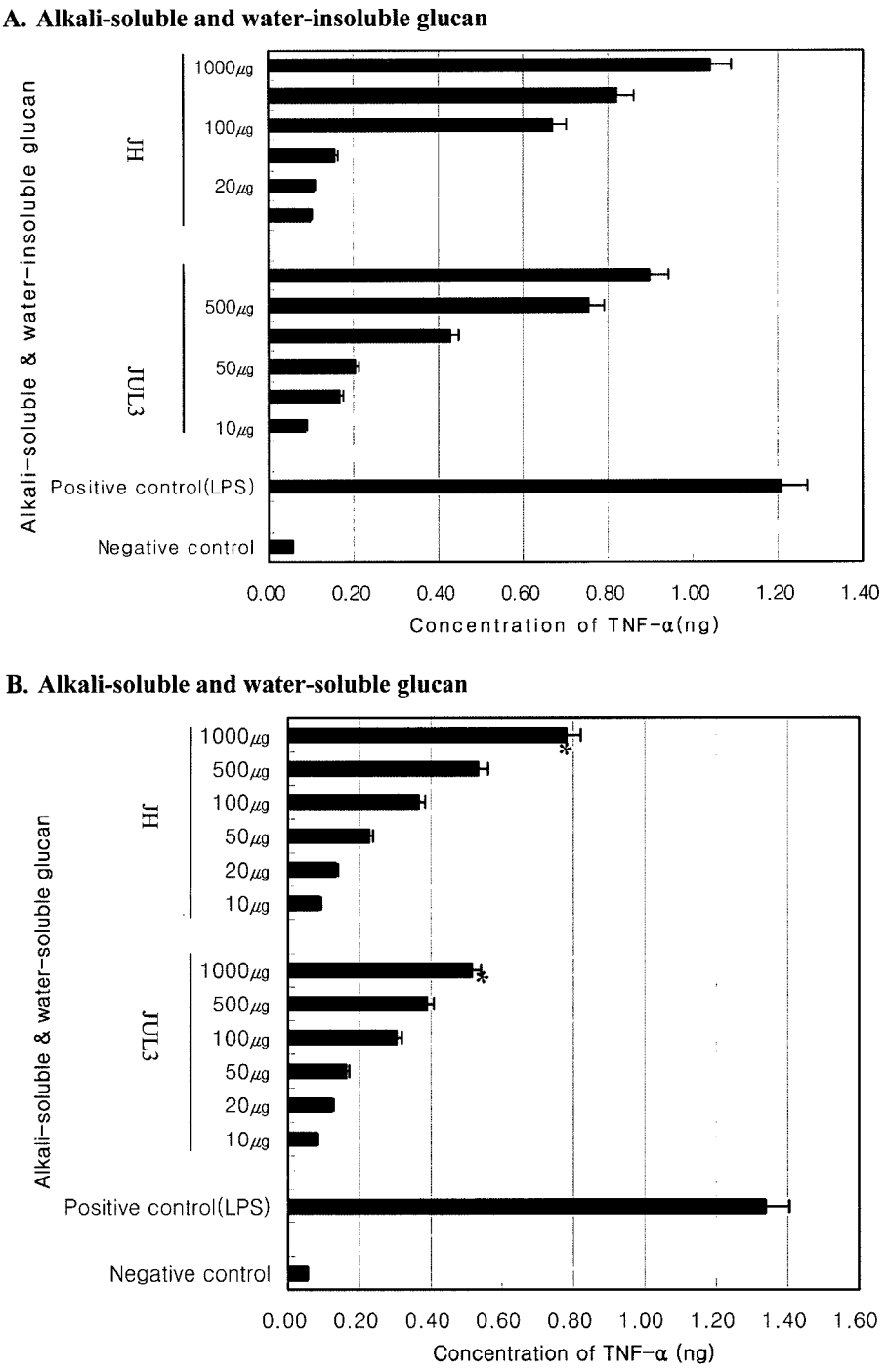


Fig. 3. Effect of gradient injection of the β-glucans on TNF-α secretion of murine macrophage cell line, Raw 264.7.
Different doses (10, 20, 50, 100, 500, 1,000 μg) of the β-glucans (alkali-soluble and water-insoluble glucan, and alkali-soluble and water-soluble glucan) were added to Raw 264.7 (4×10^5 cells/ml) cells and incubated for 21 hours. The supernatants were harvested, and the amount of TNF-α was measured using L929 bioassay. LPS was used as a positive control. The results represent the mean of three independent experiments±SD. *Represents significant difference between wild-type (JH) and mutant (JUL3) at $P<0.05$ level.

other sources, it is clearly important to determine the biological characteristics of any given glucan before its use in any clinical practice. The results of the current study indicate that the alkali-soluble and water-soluble glucan exhibit a high degree of phagocytic activation and moderate TNF-α secretion, compared with that by the alkali-soluble and water-insoluble glucan. TNF-α activity was not suppressed to any appreciable degree by alkali-soluble

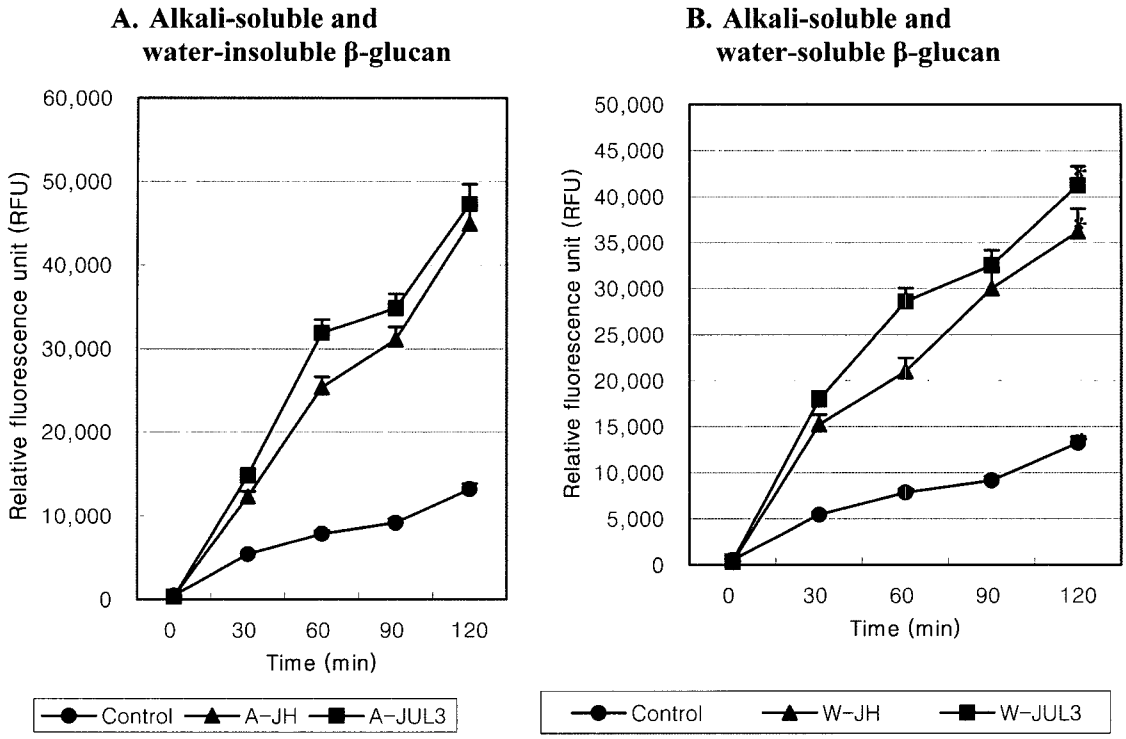


Fig. 4. Potentiation of β-glucans on phagocytosis by murine macrophage cell line, RAW 264.7.
Murine macrophage cell line, RAW 264.7, was stimulated with β-glucans. FITC-labeled *E. coli* was added to RAW 264.7 at a ratio of 1:20 (macrophage : bacteria). The results represent the mean of three independent experiments±SD. *Represents significant difference between wild-type (JH) and mutant (JUL3) at $P<0.05$ level.

glucans. With regard to the biological implications of the above observations, we found that the intermediate concentration of alkali-soluble glucan resulted in the release of nitric oxide from macrophages, but alkali-soluble glucan stimulated the release of TNF-α, regardless of the concentration. The recognition of alkali-soluble glucan by macrophages constitutes an important mechanism inherent to the interaction of the host with various pathogens [8]. Alkali-soluble glucan was shown to increase the number and function of intraepithelial lymphocytes in the intestine when orally administered [32], suggesting that alkali-soluble glucan might efficiently be absorbed by the intestines. Moreover, the alkali-soluble and water-soluble glucan used in this study was a type that is extremely water-soluble. Therefore, when orally administered, this alkali-soluble and water-soluble glucan probably entered the circulation after intestinal absorption, and would be able to activate intrapulmonary lymphocytes and macrophages. However, in this study, we did not measure alkali-soluble and water-soluble glucan levels in the blood after oral administration. Alkali-soluble and water-soluble glucan would be a valuable component in a therapeutic approach to prevent and treat oral bacterial infections by upregulation of host antibacterial defense mechanisms. Furthermore, these glucans may also prove to be valuable additives in food processed products or cosmetics.

Acknowledgment

This work was supported by Biogreen 21 of Rural Development Administration, Republic of Korea (#2005-0108).

References

1. Chorvatovicova J. Sandula and G. Kogan. 1999. Protective effect of the yeast glucomannan against cyclophosphamide-induced mutagenicity. *Mutat. Res.* **444:** 117–122.
2. Czop, J. K. and J. Kay. 1991. Isolation and characterization of glucan receptors on human mononuclear phagocytes. *J. Exp. Med.* **173**: 1511–1518.
3. Ding, A. H. and D. J. Stuehr. 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J. Immunol.* **141:** 2407–2412.
4. Esteban, A. and J. Meseguer. 2004. Glucan receptor but not mannose receptor is involved in the phagocytosis of *Saccharomyces cerevisiae* by seabream (*Sparus aurata* L.) blood leucocytes. *Fish Shellfish Immunol.* **16:** 447–451.
5. Fleet, G. H. and D. J. Manners. 1976. Isolation and composition of an alkali-soluble glucan from the cell walls of *Saccharomyces*. *J. Gen. Microbiol.* **94:** 180–192.

6. Ha, C. H., Y. T. Kim, S. T. Lim, C. W. Kim, and H. I. Chang. 2002. Analysis of alkali-soluble glucan produced by *Saccharomyces cerevisiae* wild-type and mutants. *Appl. Microbiol. Biotechnol.* **58:** 370–377.
7. Hong, F., J. Yan, D. J. Allendorf, J. T. Baran, G. R. Ostroff, and G. D. Ross. 2003. Beta-glucan functions as an adjuvant for monoclonal antibody immunotherapy by recruiting tumoricidal granulocytes as killer cells. *Cancer Res.* **63:** 9023–9031.
8. Hoffman, O. A. and A. H. Limper. 1993. *Pneumocystis carinii* stimulates tumor necrosis factor-alpha release from alveolar macrophages through a beta-glucan-mediated mechanism. *J. Immunol.* **150:** 3932–3940.
9. Ito, H., S. Kuroda, H. Sakamoto, J. Kajihara, T. Kiyota, H. Hayashi, M. Kato, and M. Seko. 1986. Molecular cloning and expression in *Escherichia coli* of the cDNA coding for rabbit tumor necrosis factor. *DNA* **5:** 149–156.
10. Jamas, S., C. K. Rha, and A. J. Sinskey. 1986. Morphology of yeast cell wall as affected by genetic manipulation of (1-6) glycosidic linkage. *Biotech. Bioeng.* **28:** 769–784.
11. Jamas, S., C. H. Osten, A. J. Sinskey, and C. K. Rha. 1990. Spectral analysis of glucan produced by wild-type and mutant *Saccharomyces cerevisiae*. *Carbohydr. Polym.* **13:** 207–219.
12. Jung, K., Y. Ha, S. K. Ha, D. U. Han, D. W. Kim, W. K. Moon, and C. Chae. 2004. Antiviral effect of *Saccharomyces* cerevisiae beta-glucan to swine influenza virus by increased production of interferon-gamma and nitric oxide. *J. Vet. Med. B. Infect. Dis. Vet. Public Health* **51:** 72–76.
13. Kollar, R., B. B. Reinhold, E. Petrakova, H. J. Yeh, G. Ashwell, J. Drgonova, J. C. Kapteyn, F. M. Klis, and E. Cabib. 1997. Architecture of the yeast cell wall. Beta(1→6)-glucan interconnects mannoprotein, beta(1→)3-glucan, and chitin. *J. Biol. Chem.* **272:** 17762–17775.
14. Kramer, S. M. and M. E. Carver. 1986. Serum-free *in vitro* bioassay for the detection of tumor necrosis factor. *J. Immunol. Methods* **93:** 201–208.
15. Kozel, T. R., A. Percival, and Q. Zhou. 2004. Biological activities of naturally occurring antibodies reactive with *Candida albicans* mannan. *Infect. Immun.* **72:** 209–218.
16. Kurihara, K., N. N. Miura, S. Horie, Y. Usui, Y. Adachi, T. Yadomae, and N. Ohno. 2003. Effect of CAWS, a mannoprotein-beta-glucan complex of *Candida albicans*, on leukocyte, endothelial cell, and platelet functions *in vitro*. *Biol. Pharm. Bull.* **26:** 233–240.
17. Lee, J. N., I. H. Ji, G. E. Kim, H. N. Kim, J. W. Sohn, S. D. Kim, and C. W. Kim. 2001. Purification of soluble beta glucan with immune enhancing activity from the cell wall of yeast. *Biosci. Biotechnol. Biochem.* **65:** 837–841.
18. Luzio, S. 1961. Identification of a reticuloendothelial stimulating agent in zymosan. *Am. J. Physiol.* **200:** 297–303.
19. Ohmura, Y., I. Motokawa, K. Sukarai, and T. Ando. 2001. Protective effects of a protein-bound polysaccharide, PSK, on *Candida albicans* infection in mice via tumor necrosis factor induction. *Int. Immunopharmacol.* **1:** 1797–1811.
20. Ohno, N., T. Hashimoto, Y. Adachi, and T. Yadomae. 1996. Effect of glucans on the nitric oxide synthesis by peritoneal macrophage in mice. *Biol. Pharm. Bull.* **19:** 608–612.
21. Price, C. W., G. B. Fuson, and H. J. Phaff. 1978. Genome comparison in yeast systematics: Delimitation of species within the genera *Schwanniomyces*, *Saccharomyces*, *Debaryomyces*, and *Pichia*. *Microbiol. Rev.* **42:** 161–167.
22. Paulik, S., S. Svrcek, M. Huska, J. Mojzisova, A. Durove, and Z. Benisek. 1992. The effect of fungal and yeast glucan and levamisole on the level of the cellular immune response *in vivo* and leukocyte phagocytic activity in mice. *Vet. Med. (Praha)* **37:** 675–681.
23. Ross, G. D., J. Yan, Y. Xia, and J. Vetvickova. 1999. Therapeutic intervention with complement and β-glucan in cancer. *Immunopharmacology* **42:** 61–74.
24. Sakurai, T., N. Ohno, and T. Yadomae. 1999. Effects of fungal glucan and interferon on the secretory functions of murine alveolar macrophages. *J. Leuk. Biol.* **60:** 118–124.
25. Soltys, J., P. Dubinsky, O. Tomasovicova, H. Auer, and H. Aspock. 1996. Effect of glucan immunomodulator on the immune response and larval burdens in mice with experimental toxocarosis. *Appl. Parasitol.* **37:** 161–167.
26. Sakurai, T., N. Ohno, and T. Yadomae. 1994. Changes in immune mediators in mouse lung produced by administration of soluble (1→3)-beta-D-glucan. *Biol. Pharm. Bull.* **17:** 617–624.
27. Stephen, X. R. A. Ruckmana, Joseph, F. Borzellecac, Chad, and B. Sanduskyd. 2004. Toxicological and metabolic investigations of the safety of N-a-Lauroyl-l-arginine ethyl ester monohydrochloride (LAE). *Food Chem. Toxicol.* **42:** 245–259.
28. Schnyder, J. and M. Baggiolini. 1978. Role of phagocytosis in the activation of macrophages. *J. Exp. Med.* **148:** 1449–1454.
29. Schnyder, J. and M. Baggiolini. 1978. Secretion of lysosomal hydrolases by stimulated and nonstimulated macrophages. *J. Exp. Med.* **148:** 435–439.
30. Schnyder, J. and M. Baggiolini. 1980. Induction of plasminogen activator secretion in macrophages by electrochemical stimulation of the hexose monophosphate shunt with methylene blue. *Proc. Natl. Acad. Sci. USA* **77:** 414–419.
31. Tsikitis, V. L., J. E. Albina, and J. S. Reichner. 2004. Beta-glucan affects leukocyte navigation in a complex chemotactic gradient. *Surgery* **136:** 384–389.
32. Tsukada, C., H. Yokoyama, C. Miyaji, Y. Ishimoto, H. Kawamura, and T. Abo. 2003. Immunopotentiation of intraepithelial lymphocytes in the intestine by oral administrations of beta-glucan. *Cell Immunol.* **221:** 1–5.
33. Vassallo, R. and A. H. Limper. 2000. Isolated *Pneumocystis carinii* cell wall glucan provokes lower respiratory tract inflammatory responses. *J. Immunol.* **164:** 3755–3763.
34. Van Berkel, M. A., R. C. Montijn, and F. M. Klis. 1994. Glycosylation of chimeric proteins in the cell wall of *Saccharomyces cerevisiae*. *FEBS Lett.* **349:** 135–138.
35. Williams, D. L., R. B. McNamee, E. L. Jones, H. E. Ensley, and I. W. Browder. 1992. Development of a water-soluble, sulfated (1→3)-beta-D-glucan biological response modifier derived from *Saccharomyces cerevisiae*. *Carbohydr. Res.* **235:** 247–257.