Food Sci. Biotechnol. Vol. 17, No. 3, pp. 683 ~ 686 (2008)

RESEARCH NOTE

Food Science and Biotechnology

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Effect of Amination on the Biological Activity of β-Glucan from *Sangwhang* (*Phellinus linteus*)

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Abstract β-Glucans, which were isolated from *sangwhang* (*Phellinus linteus*), were subjected to reductive amination and the biological properties of the derivative were investigated. The degree of substitution of the aminated *sangwhang* β-glucan was calculated by elemental analysis to be 1.13. Bronchoalveolar lavage (BAL) experiments showed that the aminated derivative increased nitric oxide production. In addition, the amination enhanced *in vitro* cytotoxic activities against HT1080 and SNU-C2A cell lines. Thus, the aminated derivative is shown to enhance immune systems by the incorporation of amino groups into the polymer structure.

Keywords: *sangwhang*, β-glucan, amination, nitric oxide, cytotoxic activity

Introduction

Edible mushrooms have been enjoyed worldwide due to their nutritional benefits as well as culinary characteristics (1,2). In addition, mushrooms are regarded as a health food since they contain bioactive polymers which contribute to various biological properties. Therefore, much consideration has recently focused on the bioactive polymers of mushrooms as medicinal agents for anti-cancer (3), hypolipidemia (4), and hypoglycemic effect (5).

Phellinus linteus, named '*sangwhang*' in Korea, is a well-known mushroom, which has attracted great attention due to its physiological activities including its anti-tumor effect (6). (1-3)-β-Glucan with (1-6)-linkage, one of the most active polysaccharides in *sangwhang*, is shown to have beneficial effects on tumor growth inhibition through the stimulated proliferation of T-lymphocytes, B-lymphocytes, and macrophages (7-11). Besides immunomodulating activity, *sangwhang* originated β-glucan has a positive effect on control of inflammatory (12,13), diabetes mellitus (14), and hyperplasia (15).

As one of the methods to enhance the biological properties of polysaccharides, chemical modification has been extensively used for commercial and scientific interests. The incorporation of new functional groups by the chemical modification causes the structural changes of polysaccharides, consequently affecting their biological properties (16). Among various chemical modifications, amination produces the derivative that generally exhibits the most stability against hydrolysis and thermolysis and enhanced physical properties (17). Previously, β-glucans from cereal sources such as oats have been chemically modified with amination, affecting the biological activities such as bile acid binding capacity, anti-microbial effect, and the synthesis of nitric oxide (18). However, the amination of β-glucan from *sangwhang* has never been reported yet to our best knowledge.

Thus, in this study, the β-glucan extracted from *sangwhang* was modified by reductive amination and its biological properties such as nitric oxide production and cytotoxic activities were investigated.

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Received November 20, 2007; Revised December 3, 2007;
Accepted December 3, 2007

Materials and Methods

Isolation and modification Based on a previous method (6), β-glucan was extracted from *sangwhang* and subjected to chemical modification. According to the method of Yalpani (19), the β-glucan (1.0 g) in dimethyl sulfoxide (DMSO, 40 mL) was mixed with paraformaldehyde (2 g) and consecutively stirred at 90°C for 3 hr, at 125°C for 1 hr, and at 135°C for 1 hr. The resulting solution was cooled to room temperature and treated with acetic anhydride (4 mL) for 20 hr in the dark. After the oxidized product was precipitated with methanol (40 mL), it was treated with sodium acetate (3.6 g) and sodium cyanoborohydrate (2.2 g) in DMSO (100 mL) for 7 days at room temperature. The resulting product was dialyzed against distilled water for 4 days and lyophilized. The degree of substitution was measured using an elemental analyzer (EA1110; CE Instruments, Milano, Italy).

Biological properties To evaluate the effect of amination on nitric oxide production, bronchoalveolar lavage (BAL) cells (5.0×10^5 cells/mL) obtained by the method of Shin *et al.* (6) were suspended in Dulbeccos's modified Eagle's medium (DMEM, 12-604F; Cambrex Bio Science Walkersville Inc., Walkersville, MD, USA) with 10% fetal bovine serum and 100 unit/mL penicillin/streptomycin. The cell suspension (1 mL) was transferred to 12-well tissue culture plates (Greiner, Nrtingen, Germany) and incubated at 37°C for 2 hr in 5% CO_2. After the media were replaced with phenol red-free DMEM, the native and aminated samples (10, 20, 50, and 100 μg/mL) were added and incubated for an additional 24 hr. The culture supernatant was mixed with an equal volume of Griess reagent (1%

sulfanilamide/0.1% naphthylene diamine dihydrochloride/ 2.5% H_3PO_4) (G-4410; Sigma-Aldrich Chemical Co., Milwaukee, WI, USA) at room temperature for 2 min. Nitric oxide production was measured by using sodium nitrite as a standard at 540 nm (20).

In vitro cytotoxic activities against HT1080 human fibrosarcoma cells and SNU-C2A colon adenocarcinoma cell lines (Korean Cell Line Bank, Seoul, Korea) were investigated by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (M-5655; Sigma-Aldrich Chemical Co.) (21). The cells were grown in RPMI1640 medium (LM 011-01; JBI Welgene Co., Daegu, Korea) supplemented with 10% fetal bovine serum and 100 unit/mL penicillin-streptomycin solution (P-4458; Sigma-Aldrich Chemical Co.) at 37°C in a 5% CO_2 environment. The cells (1.0×10^4 cells/well) treated with the samples having various concentrations (50, 100, 200, and 400 μg/mL) were incubated for 92 hr. The MTT solution was added and further incubated for 4 hr. After dissolving formazan crystals by adding DMSO, the optical density of the formazan solutions was measured on the multi-well enzyme-linked immunosorbent assay (ELISA) automatic spectrometer reader (ELx800UV; Bio-Tek Instrument Inc., Windoski, VT, USA) at 540 nm.

Statistical analysis All experiments were carried out in triplicate. Statistical analysis was performed with one-way analysis of variance in SPSS 12.0 (SPSS Inc., Chicago, IL, USA). Significance of difference among samples was estimated by Duncan's multiple range test at the level of 5%.

Results and Discussion

Identification of aminated derivative The polysaccharide isolated from *sangwhang* was proven to contain (1-3)-β-glucan with (1-6)-linkage (6) and this was subjected to reductive amination.

Degree of substitution (DS) of the aminated polysaccharide per glucose unit was determined based on the nitrogen percentage by elemental analysis, according to the following equation;

$$DS=\frac{162\times N\%/14}{100-(13/14\times N\%)}$$

The elemental analysis of the derivative was 45.51% of carbon, 5.74% of hydrogen, and 8.97% of nitrogen and the calculated DS was 1.13. Thus, the elemental analysis data confirmed that the aminated derivative having 1.13 of DS was successfully produced, showing the existence of amine groups in the derivative.

Effect of amination on nitric oxide production It is well known that edible mushrooms have an anti-tumor effect, macrophage stimulation, and other various biological properties which are attributed to the branched β-glucans in the mushrooms (21-23). The β-glucan-mediated anti-tumor effect is related to the activation of macrophages via interaction with β-glucan specific receptors. The activated macrophages release active substances such as nitric oxide which is produced by nitric oxide synthase, increasing protection against infections (23-26).

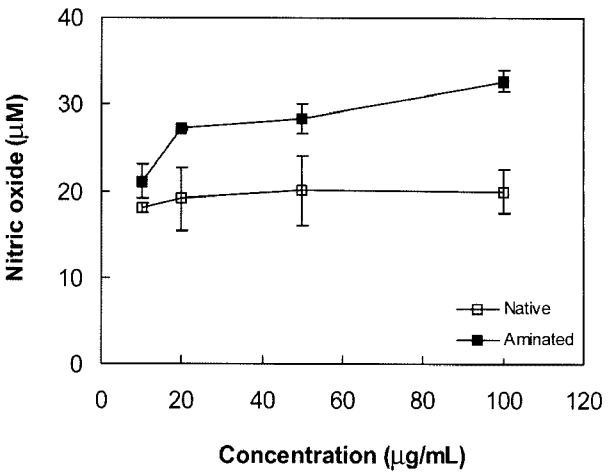

Fig. 1. Production of nitric oxide by BAL cells treated with native and aminated *sangwhang* β-glucans. The error bars indicate standard deviation.

The effect of native and aminated *sangwhang* β-glucans on nitric oxide production is shown in Fig. 1. The aminated derivative significantly generated more nitric oxide than the native sample at all concentrations ($p<0.05$). Furthermore, the derivative produced nitric oxide in a concentration-dependent manner. Specifically, the amount of the produced nitric oxide increased 1.6-fold at a concentration of 100 μg/mL.

Shin *et al.* (18) also exhibited 2.6-fold increase in the nitric oxide production of the aminated oat β-glucan, which was favorably compared to our results. They demonstrated that this developed nitric oxide synthesis would be due to the substitution of hydroxyl groups by amino groups in β-glucan. Therefore, the introduction of amino groups into the polymer chain might contribute to improved interaction with the specific β-glucan receptors, adequately stimulating nitric oxide production by activated macrophages.

Effect of amination on tumor cell growth inhibition Figure 2 presents the growth inhibition effects of native and aminated *sangwhang* β-glucan against HT1080 human fibrosarcoma cells and SNU-C2A colon adenocarcinoma cells. Even though both samples had inhibitory effects against 2 tumor cell lines, more improved growth inhibition was observed in the aminated derivative. Specifically, inhibitory activity by amination against SNU-C2A cells increased 1.5-fold at a concentration of 400 μg/mL. Thus, these results demonstrated that the introduction of amino groups into the structure of β-glucan effectively suppressed the growth of the tumor cells. In addition, macrophages' stimulation by the aminated derivative, which is mentioned in Fig. 1, might also contribute to its enhanced cytotoxic activity against the tumor cells.

It is well known that the (1-3)-(1-6)-β-D-glucans have cytotoxic effects against tumor cells and these activities can be improved by chemical modification of polymers (6,23,27-30), which was also confirmed in this study.

Furthermore, the observation of greater cytotocixity against HT1080 cell implied that the inhibitory effect of the β-glucan samples seemed to be specific for particular tumor cell lines.

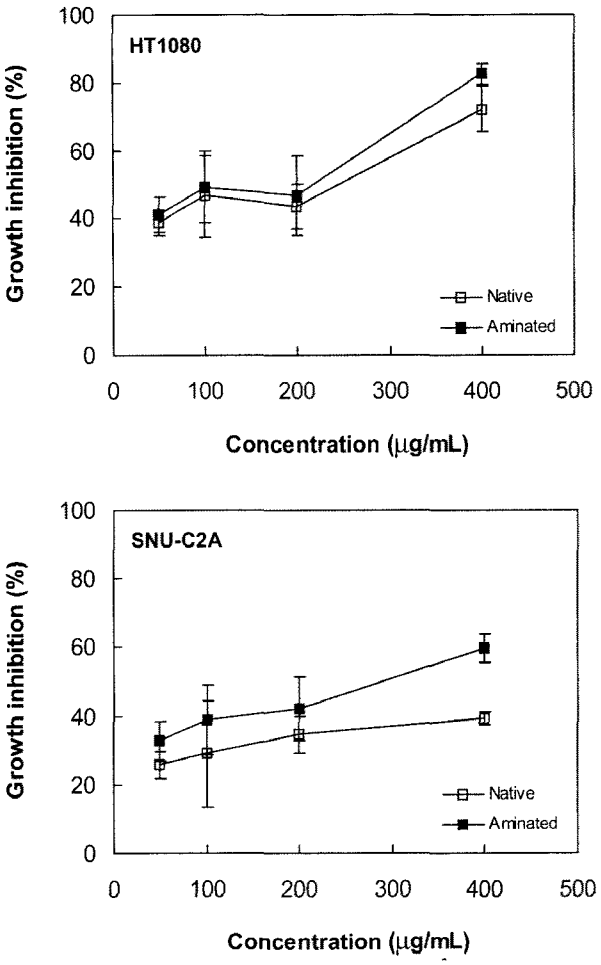

Fig. 2. Growth inhibition effects of native and aminated *sangwhang* β-glucans on HT1080 (human null fibrosarcoma) and SNU-C2A (human colon adenocarcinoma) cells. The error bars indicate standard deviation.

In conclusion, the β-glucan extracted from *sangwhang* was modified by reductive amination, which improved physiological activities involved in immune system. These results indicated that the aminated derivative exhibited the improvement of nitric oxide production and *in vitro* growth inhibitory effects against HT1080 and SNU-C2A cells.

Acknowledgments

This study was supported by a grant of the Korea 21 R&D project, Ministry of Health & Welfare, Korea (A030156).

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