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Biological activities of the polysaccharides produced from submerged culture of the edible Basidiomycete *Grifola frondosa*

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Abstract

Five groups of polysaccharides were prepared from mycelium extract and top and bottom fraction of filtrate precipitates by submerged culture of *Grifola frondosa* at two different media (glucose and PMP medium) and their individual biological activities were studied. These polysaccharides had diverse molecular mass (470–1650 kDa) and different biological activities at the concentrations of 0.01–0.2% (w/v). Most of polysaccharides had antioxidant and free radical scavenging activities after UV irradiation, where *G-2* (bottom fraction of filtrate precipitates from glucose medium, MW 770 kDa) and *G-3* polysaccharide (mycelium extract from glucose medium, MW 500 kDa) showed strong activity. The *P-1* (from top fraction of filtrate precipitates from PMP medium, MW 1650 kDa) and *P-3* polysaccharide (from mycelium extract from PMP medium, MW 470 kDa) increased the proliferation of fibroblasts by approximately 23–25%. Other two groups of polysaccharides produced from glucose medium (*G-2* and *G-3* polysaccharides) showed also notable proliferation activity for fibroblasts. Treatment of fibroblasts with *P-3* polysaccharide significantly increased the biosynthesis of collagen by approximately 80%. *G-2* and *G-3* polysaccharides showed also marked activity. However, *G-1* and *P-1* polysaccharides had only negligible activity in collagen biosynthesis.

Keywords: Antioxidant activity; Collagen biosynthesis; Fibroblasts; Free radicals; *Grifola frondosa*; Mushroom; Polysaccharides

1. Introduction

Grifola frondosa is a *Basidiomycete* fungus belonging to the order *Aphyllopherales*, and family *Polyporaceae*. Fruit body and liquid-cultured mycelium of this mushroom have been reported to contain useful antitumor polysaccharides from various fractions. These polysaccharides have been identified as many types of glucans (e.g. β-1,6- and β-1,3-) [20,22,23].

Wide varieties of applications of β-glucan have been reported, including thickening and stabilizing agents in chemical industries, and immunostimulating and antitumor agents in clinical uses [5,27,28]. Apart from these applications, β-glucan has been used as a substance that enhances the skin's natural ability to heal and protect itself against infection [17].

β-Glucan now in use as a component of various cosmetics is mainly produced from *Saccharomyces cerevisiae* as a water-soluble particulate or its chemically modified soluble forms such as carboxymethyl or phospholyated glucan [5].

Another β-glucan, schizophyllan, which is produced from *Schizophyllum commune*, has been used as an immunotherapeutic agent for cancer treatment in Japan since 1986. Clinical studies have demonstrated that this glucan activates macrophage, subsequently increase the T-cell cascade, which mean increasing the body's immune defense mechanism [28]. This applies as much as to the skin as to any other aspect of the immune defenses. It has been already reported that schizophyllan has strong immunostimulating activity. In this regard, schizophyllan has been regarded as a good candidate for blocking the skin aging process. However, there have been difficulties in using schizophyllan in cosmetics due to its high production cost [27].

Several different kinds of polysaccharides have been produced from liquid culture of mushrooms and their diverse physiological activities have been elucidated. However, their applications have been concentrated on mainly medicinal uses such as antitumor and immunostimulating agents [10,14,24,25,29].

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In this study, in an attempt to search for functional cosmetic ingredients from mushrooms, we have produced five groups of polysaccharides by submerged culture of *G. frondosa* and investigated their diverse biological activities including antioxidant activity, free radical scavenging activity after UV irradiation, proliferation of the fibroblasts, and collagen biosynthesis activity.

To the best of our knowledge, this is the first report on the wide application of polysaccharides produced from submerged culture of mushrooms to cosmeceuticals.

2. Materials and methods

2.1. Microorganism and media

A culture of *G. frondosa* was from our culture collection isolated from mountainous district in Korea. The stock culture was maintained on potato dextrose agar (PDA) slant. Slants were incubated at 25 °C for 7 days and then stored at 4 °C. The seed culture was grown in a 250 ml flask containing 50 ml of either synthetic medium (hereafter called glucose medium; 30 g/l glucose, 6 g/l yeast extract, 2 g/l polypeptone, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.5 g/l K_2HPO_4, and 0.2 g/l $MnSO_4 \cdot 5H_2O$) or complex medium (hereafter called PMP medium; 24 g/l potato dextrose broth, 10 g/l malt extract, and 1 g/l peptone) at 25 °C on a rotary shaker incubator at 120 rpm for 7 days.

2.2. Fermentations

G. frondosa was initially grown on PDA medium in a petridish, and then transferred to the seed culture medium by punching out 5 mm of the agar plate culture with a sterilized self-designed cutter. The seed culture was grown in a 250 ml flask containing 50 ml of basal medium at 25 °C on a rotary shaker incubator at 150 rpm for 4 days. The second flask culture experiments were performed in a 500 ml flask containing 100 ml of the media after inoculating with 3% (v/v) of the seed culture under the conditions described above. The fermentation medium was inoculated with 3% (v/v) of the seed culture and then cultivated at 25 °C in a 5-l stirred-tank fermenter (Best Korea Co. Ltd., Daejeon, Korea). Fermentations were conducted under the conditions of temperature 25 °C, aeration rate 2 vvm, agitation speed 150 rpm, pH 5.5, and working volume 3 l. The seed culture was transferred to the fermentation medium and was cultivated for 13 days. All experiments were performed at least in duplicates.

2.3. Preparation of polysaccharides

The fermentation broth was centrifuged at 8000 × *g* for 20 min, and the resulting supernatant was filtered (Millipore, 0.45 μm) and mixed with four times volume of absolute ethanol, stirred vigorously and left overnight at 4 °C. The top fraction of polysaccharides in filtrates were pooled and designated it as *G-1* polysaccharide (produced from glucose medium) and *P-1* polysaccharide (produced from PMP medium), respectively, while the precipitated exo-polysaccharide (bottom fractions) was collected after centrifugation and designated it as *G-2* polysaccharide. No bottom fraction of polysaccharide was formed in case of PMP medium (i.e. *P-2* polysaccharide was not available). To extract intracellular polysaccharides, the mycelial biomass was submerged in hot water for 4 h at 100 °C followed by extracted with ethanol for 24 h at 4 °C, and the resulting polysaccharides from glucose and PMP medium were named as *G-3* and *P-3* polysaccharide, respectively (Fig. 1).

2.4. Analytical methods

2.4.1. Estimation of mycelial growth and polysaccharide production

Samples collected at various intervals from fermentation broth were centrifuged at 8000 × *g* for 20 min, and the resulting supernatant was filtered through membrane filtration (Millipore, 0.45 μm). The resulting culture filtrate was mixed with four times volume of absolute ethanol, stirred vigorously and left overnight at 4 °C. The precipitated exo-polysaccharides were centrifuged at 8000 × *g* for 10 min, discarding the supernatant. The precipitate of exo-polysaccharide was lyophilized and the weight of the polymer was estimated. Dry weight of mycelium was measured after repeated washing of the mycelial pellets with distilled water and drying overnight at 70 °C to a constant weight.

2.4.2. Molecular weight determination

The molecular weight of polysaccharides was estimated based on a calibration curve made by HPLC (Waters Co., Milford, MA, USA) with Shodex OHpak KB-804 column (0.8 cm × 30 cm) (Showa Denko K.K., Tokyo, Japan) using distilled water as a mobile phase (column temperature, 50 °C; flow rate, 0.8 ml/min; injection volume, 20 μl). The eluate was monitored by an evaporative light scattering detector (ELSD, Alltech Associates, Deerfield, IL, USA). Various dextrans of diverse molecular mass were used as standard molecules (Polymer Standards Service Inc., Silver Spring, MD, USA).

2.4.3. Antioxidant activity

Superoxide dismutase (SOD) activity was measured using xanthine–xanthine oxidase system as a source of superoxide and nitroblue tetrazolium (NBT) as a scavenger for this radical. One unit of SOD activity was defined as the amount of enzyme causing 50% inhibition of the initial rate of reduction of NBT. SOD activity was determined as described by Beauchamp and Fridovich [1] by measuring percent inhibition of NBT reduced by SOD. Xanthine oxidase was used to generate O_2^- during the conversion of xanthine to uric acid. One ml of 0.3 mM xanthine, 500 μl of 0.6 mM EDTA, 500 μl of 0.15 mM NBT and 100 μl of polysaccharides were mixed in the plate. After adding

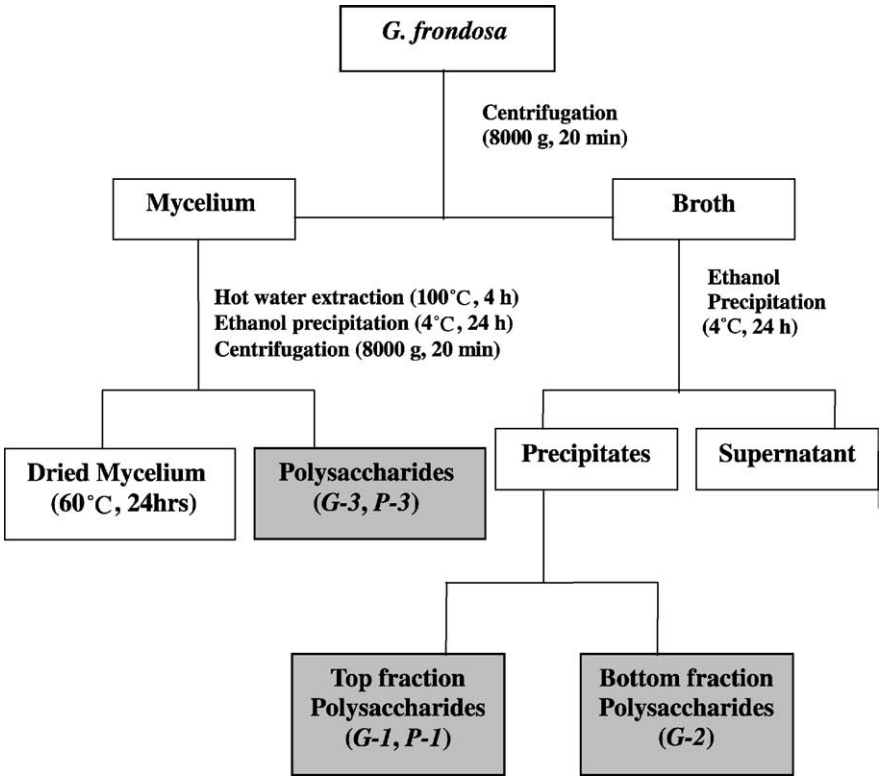

Fig. 1. Recovery process of five groups of polysaccharides produced by submerged culture of *Grifola frondosa*.

xanthine oxidase, the formazan produced was measured spectrophotometrically at 560 nm. The plates were incubated for 20 min at 37 °C. Experiments were performed in triplicate and their mean values were noted.

2.4.4. Measurement of free radical scavenging activity after UV irradiation

Human dermal fibroblasts (HDF) were cultured on Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (100 μg/ml), and then 2×10^5 cell per well were added on a 24-well microtiter plate. The cells were maintained in a humidified 5% CO_2–95% air incubator at 37 °C. Analyses of confluent fibroblast cultures were carried out at 6–10 passages of sub-cultivation. The cultured fibroblast cells were treated with 0.5% polysaccharides for 1 h before UV irradiation. Immediately before UVA irradiation, the medium was replaced by phosphate-buffered saline (PBS). UVA irradiation doses were 360 mJ/cm^2. 2′,7′-Dichlorodihydrofluoresceink diacetate (DCDHF), a non-fluorescent compound, is able to react with free radical compound, especially with hydrogen and to generate fluorescent DCDHF. Cells were loaded with 5 μM DCDHF and 2% Pluronic F-127 in HCSS solution containing (120 mM NaCl, 5 mM KCl, 1.6 mM $MgCl_2$, 2.3 mM $CaCl_2$, 15 mM glucose, 20 mM HEPES, 10 mM NaOH) for 20 min at 37 °C and washed three times with HCSS solution, and the fluorescence signal of DCF ($E_x = 490$ nm; $E_m = 510$ nm), the oxidation product of DCDHF-DA by free radicals, was analyzed flow by a cytometry (Becton Dickinson, NJ, USA) [15]. Experiments were performed in triplicate and their mean values were noted.

2.4.5. Activity of proliferation of fibroblasts

Mouse fibroblasts NIH 3T3 were cultured on DMEM containing 10% FBS and then 2×10^5 cells per well were added on a 24-well microtiter plate. After addition of 10% polysaccharides into each well, the 24-well plate was maintained at CO_2 incubator (37 °C) for 2 days. After the cultivation was completed and DMEM was removed, 0.5% 60 μl MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and 500 μl of fresh DMEM was added on a 24-well plate. Again, the plate was maintained at CO_2 incubator for 2 h to give formazan formation. The quantity of formazan produced can be regarded as an indicator of cell density or viability. After dissolving the formazan in dimethyl sulfoxide (DMSO), the absorbance at 565 nm was measured with microplate reader. The proliferation of fibroblasts was evaluated by comparing the absorbance with that of the untreated control. The cell viability was calculated using the following formula:

$$\left[\frac{OD_{565}(\text{treated}) - OD_{565}(\text{blank})}{OD_{565}(\text{control}) - OD_{565}(\text{blank})}\right] \times 100$$

Experiments were performed in triplicate and their mean values were noted.

2.4.6. Collagen biosynthesis activity

Collagen from fibroblasts was quantified by the Sirius Red. Human dermal primary fibroblast p16 were seeded in 1 ml aliquots in 24-well plate at density of 1×10^5 cells per well in DMEM containing 10% FBS. After 24 h incubation at 37 °C the medium was replaced with 1 ml DMEM containing 10% polysaccharides. After 48 h, cell culture supernatant and cell extract lyses by rapid freezing and thawing, was dried onto the plate. The plates were incubated at 37 °C for overnight (humidified) and then kept for 24 h at 37 °C. The well was filled with 1 ml of 0.1% (w/v) Sirius Red F3BA in saturated picric acid and the samples were stained for 1 h at room temperature. The plate was washed five times with 2 ml of 10 mM HCl for 10 s per wash. The collagen bound stain was then extracted with 2 ml of 0.1 M NaOH for 5 min. Absorbance was then read at 565 nm in microplate reader. Experiments were performed in triplicate and their mean values were noted.

3. Results and discussion

3.1. Mycelial growth and production of polysaccharides

Fig. 2 shows the typical time profiles of mycelial growth and polysaccharide production in *G. frondosa* in a 5-l stirred-tank bioreactor. The maximum mycelial biomass and polysaccharides produced were 17 and 5.3 g/l, respectively.

3.2. Characterization of the polysaccharides

The molecular mass and the ratios of carbohydrate to protein for each polysaccharide were investigated. The polysaccharides obtained from top fraction of culture filtrates (*G-1* and *P-1* polysaccharides) had higher molecular weight than those from bottom fraction of culture filtrate (*G-2* polysaccharide) and from mycelium extracts (*G-3* and *P-3* polysaccharides) (Table 1). There was a great difference in the ratios of carbohydrate to protein among the five polysaccharides. Mycelium-extracted polysaccharide obtained from glucose medium (*G-3*) had the highest protein content (34%). The effect of this ratio on biological activities shall be discussed later.

3.3. Antioxidant activity

Reactive oxygen species (ROS) such as hydroxyl and superoxide radicals produced by sunlight, ultraviolet, chemical reactions, and metabolic processes have a wide variety of pathological effects on cellular processes [18]. Superoxide radical is one of the strongest free radicals in cellular oxidation reactions because, once it forms, it further produces various kinds of cell-damaging free radicals and oxidizing agents [7,16]. There are many diseases such as heart disease, cancer, arthritis, and the aging process itself, in which free radicals are implicated. To combat these free radicals the body needs antioxidants [7]. Antioxidant properties of four specialty mushrooms have been studied by Mau et al. [19]. They reported that almost all mushroom extracts of fruit body examined showed antioxidant activity, in which maitake (same kind of mushroom in this work) extract showed about 40% free radical inhibition activities. In the present study, antioxidant activity for five groups of polysaccharides was individually investigated by measuring the superoxide scavenging activity. As shown in Fig. 3, four groups of polysaccharides stimulated antioxidant activity, where the activity of

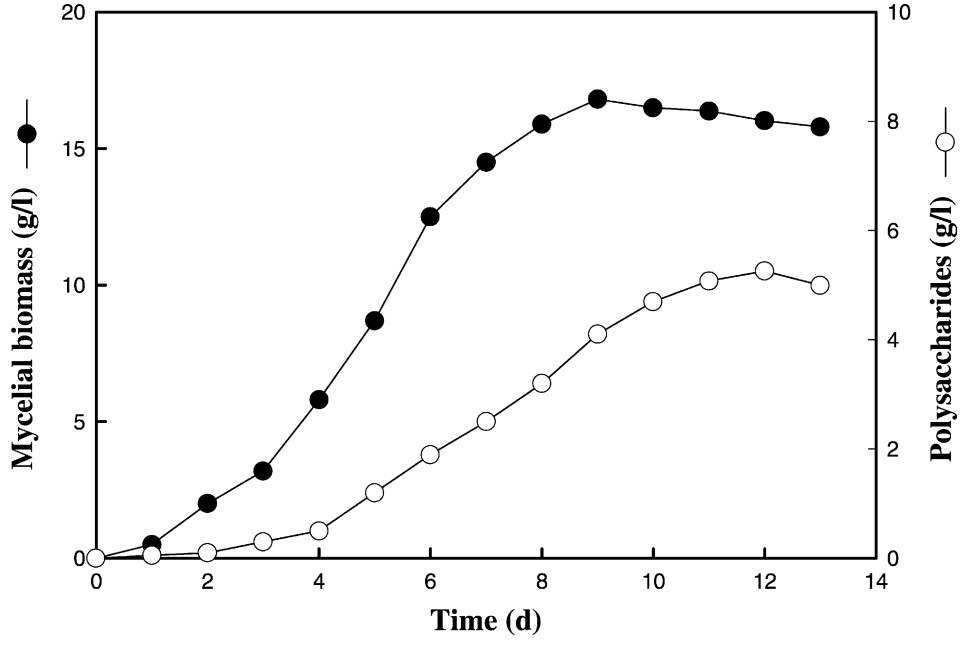

Fig. 2. Typical time profiles of mycelial growth and polysaccharide production by submerged culture of *Grifola frondosa* in a 5-l stirred-tank bioreactor.

Table 1
Characterization of five different polysaccharides produced from submerged culture of *Grifola frondosa*

Polysaccharide groups	Molecular weight (kDa)	Carbohydrates[a] (%, w/w)	Proteins[a] (%, w/w)
Top-fraction polysaccharides from culture filtrates			
At glucose medium (*G-1*)	1100	87	13
At PMP medium (*P-1*)	1650	94	6
Bottom-fraction polysaccharides from culture filtrates			
At glucose medium (*G-2*)	770	73	27
At PMP medium (*P-2*)	n/a[b]	n/a	n/a
Polysaccharide extracted from mycelium			
At glucose medium (*G-3*)	500	66	34
At PMP medium (*P-3*)	470	82	18

[a] Total carbohydrates were measured by the phenol-sulfuric acid method. Total proteins were measured by bicinchoninic acid (BCA) assay using bovine serum albumin as a standard.
[b] n/a: not available (no polysaccharide formation).

G-2 polysaccharide (MW 770 kDa) was the strongest among the five polysaccharides (90% radical inhibition). Polysaccharide from mycelium extract (*G-3* polysaccharide, MW 500 kDa) also exhibited good antioxidant activity. It is noteworthy that *G-2* and *G-3* polysaccharides have higher ratios of proteins to carbohydrates (see Table 1), which is probably closely related to higher antioxidant activity. Using these two polysaccharides, free radical scavenging activity after UV irradiation in HDF cells was further studied in vitro.

3.4. Free radical scavenging activity after UV irradiation

Free radicals are molecules or parts of molecules caused by the metabolic process of oxygen. They are hostile and damaging to cells and their functions. They can also cause a chain reaction causing the multiplication of new free radicals. Damage they cause includes interference and manipulation of protein, tissue loosening, genetic damage and the promotion of disease and aging [2].

In this study, *G-2* and *G-3* polysaccharides decreased free radicals (formed after UV irradiation) approximately by 20% at a concentration of 0.2% (w/v). However, this is relatively lower level compared to those of green-tea extract and Vitamin C, which are widely known as potent free radical scavengers (Fig. 4).

Liu et al. [15] reported extensive results on free radical scavenging activity of various mushroom polysaccharides of diverse forms (e.g. mycelium extract, fruiting body

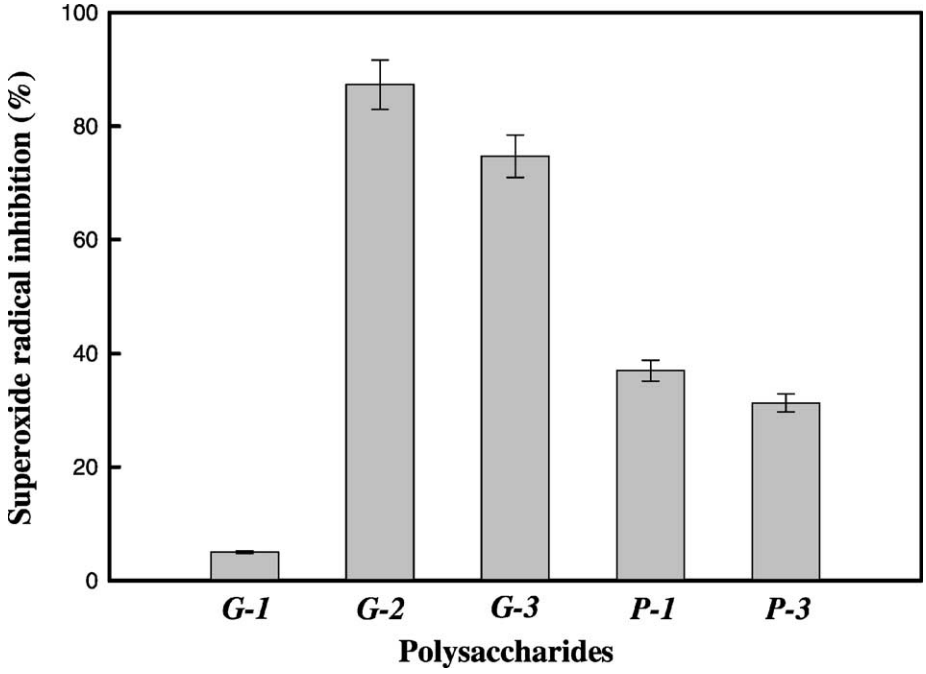

Fig. 3. Antioxidant activities for the five groups of polysaccharides produced by submerged culture of *Grifola frondosa*. The activity was measured by addition of 0.2% (w/v) polysaccharide and indicated by percentage of increase in comparison with that of control. The activity is significant ($P < 0.01$) and the values are mean ± S.E.

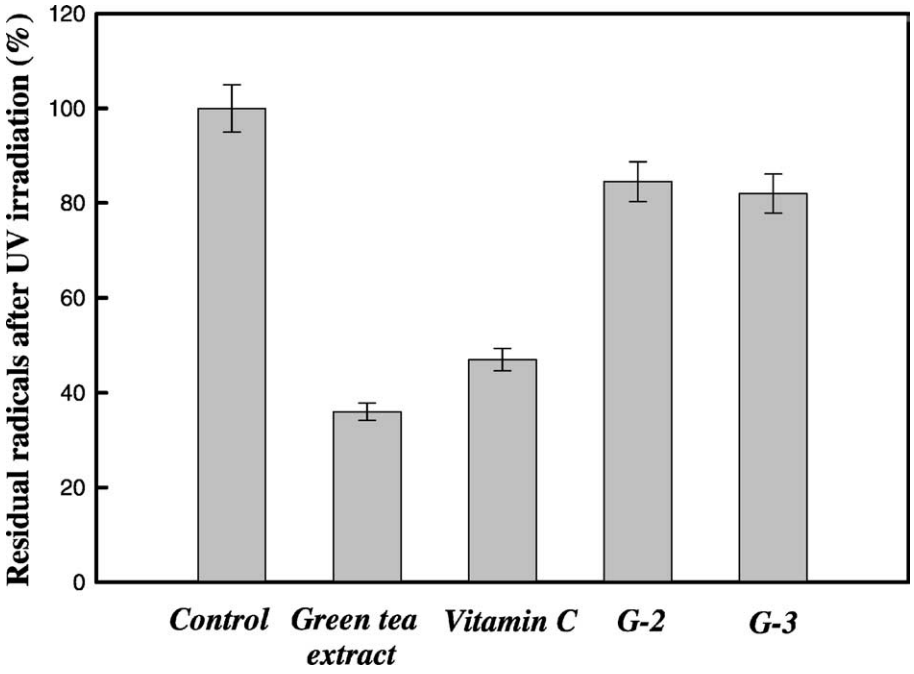

Fig. 4. Comparison of free radical scavenging activities after UV irradiation between two groups of polysaccharides produced by submerged culture of *Grifola frondosa* and green-tea extract and Vitamin C. The activity was measured by addition of 0.2% (w/v) polysaccharide. The activity is significant ($P < 0.01$) and the values are mean ± S.E.

extract, and culture filtrate). They found that five mushroom polysaccharide extracts and a protein-bound polysaccharides exhibited significant activities of superoxide and hydroxyl radical scavenging.

Other investigators have also reported that the fruit bodies of another Basidiomycete, *Ganoderma lucidum* have been evaluated as a radioprotector and antioxidant defense against oxygen radical-mediated damage. The results clearly demonstrated that the hot-water extract of *G. lucidum* showed good radioprotective ability, as well as protection against DNA damage induced by metal-catalyzed Fenton reactions and UV irradiation. They also found that the water-soluble polysaccharide isolated from the fruit body of *G. lucidum* was as effective as the hot-water extract in protecting against hydroxyl radical-induced DNA strand breaks, indicating that the polysaccharide compound is associated with the protective properties [11].

3.5. Proliferation of fibroblasts

Several applications have been reported for useful materials to skin cell proliferation.

Ohkura et al. [21] examined the effects of biscoclaurine alkaloid cepharanthine on proliferation of skin cells in vitro. They pointed out that the alkaloid was found to enhance attachment and cell layer formation of newborn rat skin epidermal cells in culture on type I collagen-coated Millipore filter, and to potentiate production of keratins in these cells. It was also found that spreading and growth of skin dermal fibroblasts were inhibited by cepharanthine in a dose-dependent manner.

According to the investigations by Bloom et al. [3], sublethal concentrations of sodium lauryl sulfate (SLS) stimulated cultured keratinocyte and fibroblast proliferation in vitro. The growth of keratinocytes, without added growth factors, continuously exposed to SLS for 4 days was stimulated approximately 89% compared to control.

In the present study, for five groups of polysaccharides, the ability to affect proliferation of skin cells was comparatively investigated at a concentration of 0.01% (w/v). As shown in Fig. 5, the *P-1* and *P-3* polysaccharides increased the proliferation of fibroblasts by approximately 23–25%, while *G-1* polysaccharide indicated negligible activity. In contrast, two groups of polysaccharides produced from glucose medium (i.e. *G-2* and *G-3* polysaccharides) showed notable proliferation activities.

Kim et al. [12] reported that β-glucan produced from *S. commune* increased the proliferation of fibroblasts by 40% at a concentration of 0.04% (w/v).

3.6. Collagen biosynthesis activity

Some types of polysaccharides have been suggested to play a possible role during the early stages of healing of a variety of connective tissues such as cell proliferation and synthesis of matrix components [8,26]. Hyaluronan (HA) has been regarded as a representative biomaterial for this use [4]. The importance of HA has been extensively described

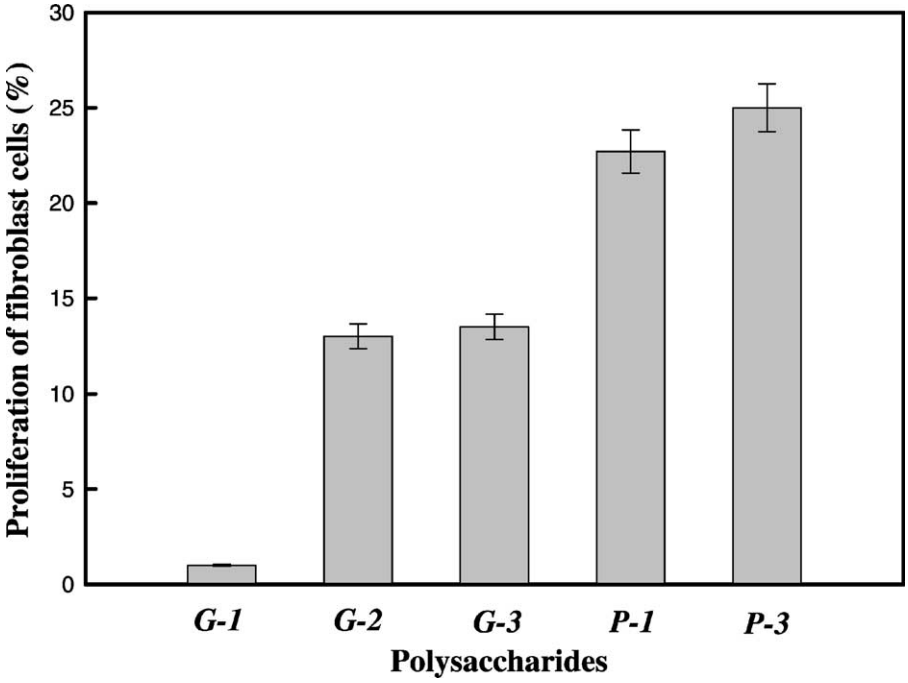

Fig. 5. Activities of proliferation of fibroblast cells for the five groups of polysaccharides produced by submerged culture of *Grifola frondosa*. The activity was measured by addition of 0.5% (w/v) polysaccharide. The activity is significant ($P < 0.01$) and the values are mean ± S.E.

for the homeostasis of connective tissues during embryogenesis and aging and its role in tissue repair. The effect of exogenous HA on the synthesis of total protein, collagen and HA by in vitro was elucidated in HDF. Data strongly indicate that a relatively high concentration of HA in the extracellular space, such as during development and in the first phases of tissue repair, would partially limit the deposition of the extracellular matrix, and of collagen in particular [4]. For this reason, HA has been widely used for an important cosmetic ingredient for a long time.

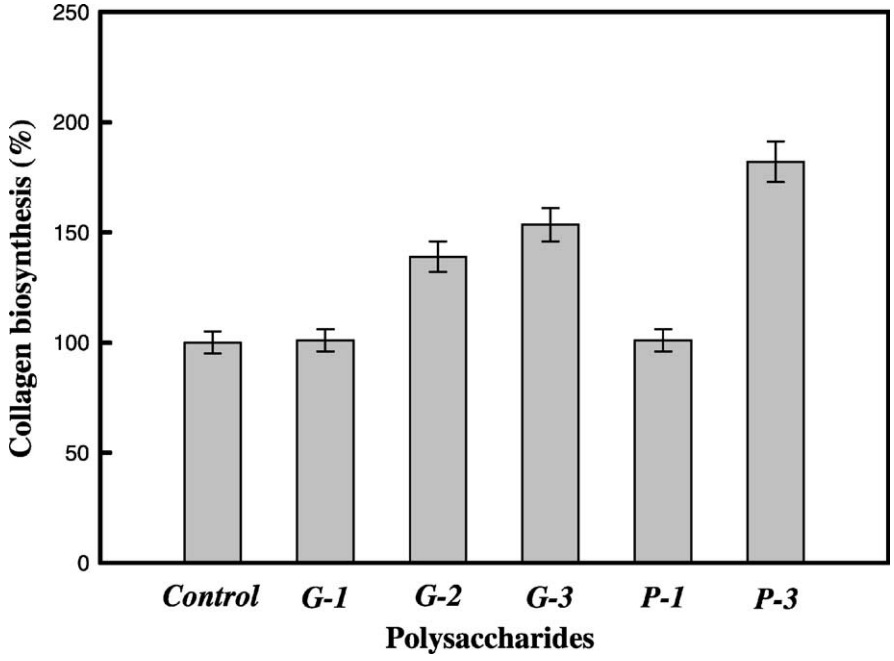

Fig. 6. Collagen biosynthesis activities for five groups of polysaccharides produced by submerged culture of *Grifola frondosa*. The activity was measured by addition of 0.05% (w/v) polysaccharide. The activity is significant ($P < 0.01$) and the values are mean ± S.E.

Recently, other types of polysaccharides have been used as alternative ingredients for enhancing collagen biosynthesis in skin cells [9,13]. For example, treatment of β-glucan produced from *S. commune* increased collagen biosynthesis by 32% at a concentration of 0.04% (w/v), whereas yeast β-glucan (from *S. cerevisiae*) gave at most a 10% increase in collagen biosynthesis [12].

More recently, it has been reported that the anabolic steroid stanozolol enhanced collagen synthesis in a dose-dependent manner in cultures of adult HDF [6]. The stimulatory effects of stanozolol on collagen synthesis were blocked by a TGF-beta-1 antisense oligonucleotide, by antibodies to TGF-beta, and in dermal fibroblast cultures derived from TGF-beta-1 knockout mice.

In the present study, the treatment of fibroblasts with *P-3* polysaccharide increased in the biosynthesis of collagen by approximately 80% at a concentration of 0.05% (w/v), while *G-2* and *G-3* polysaccharides also displayed high activity. However, two polysaccharides obtained from top fractions of culture filtrates (*G-1* and *P-1* polysaccharides) did not show any activity in collagen biosynthesis (Fig. 6). It is noteworthy that mycelium-extracted polysaccharides had higher collagen biosynthesis activity than those of other groups of polysaccharides examined.

In conclusion, we suggest that polysaccharides from *G. frondosa* can be potent ingredients for cosmetic and other biological applications.

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