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Submerged Production and Characterization of *Grifola frondosa* Polysaccharides – A New Application to Cosmeceuticals

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Summary

Grifola frondosa (maitake) is traditionally called 'the king of mushrooms' and 'the hen of the woods'. Both the fruiting bodies and the mycelium of maitake have been reported to have antitumor and antiviral activities. Recently, submerged culture processes have been developed, with the intention of providing opportunities for increased economic exploitation of maitake. Commonly the aim of these processes is to produce extracellular polysaccharides (EPS), mostly glucans, and to explore their applications, particularly in the cosmetic industry. A wide variety of EPS with different molecular chain length and chemical compositions are produced under different culture conditions. In this article, various biological and physicochemical properties of the EPS of *G. frondosa* (GF-EPS) are described, with a view to applications in the area of functional cosmeceuticals. The GF-EPS, together with GF mycelial extract (GF-MPS), showed antioxidative activity, stimulation of collagen biosynthetic activity, cell proliferation activity, and inhibitory activity of melanogenesis, without significant cytotoxicity. These diverse functionalities suggest that both GF-EPS and GF-MPS can be promising cosmetic ingredients.

Key words: cosmetic ingredients, exopolysaccharide, glucan, *Grifola frondosa* (maitake), human dermal fibroblasts, matrix metalloproteinase, skin aging

Introduction

Mushrooms have recently become attractive not only as functional foods, but also as sources of physiologically beneficial medicines. For centuries, edible mushrooms have been used to treat various health conditions, including cancer, diabetes, viral infections, and bacterial infections (*1–5*). Polysaccharides derived from mushrooms that have potent antitumor activities *in vitro* have been shown to stimulate the host immune system in animal studies. In recent clinical trials, medicinal mushrooms, or polysaccharides extracted from them, have been shown to extend the survival of cancer patients and to improve their quality of life (*6,7*).

Recently, mushroom polysaccharides have found applications beyond the pharmaceutical area. Due to their ability to accelerate collagen biosynthesis and to provide photoprotection, they have found applications as functional materials within the cosmetic industry. The polysaccharides of *Grifola frondosa* have good potential in this area (*8,9*).

G. frondosa (maitake) is a basidiomycete belonging to the order Polyporales, and the family *Meripilaceae*. The fruiting body and also the mycelium obtained from submerged liquid culture have been reported to contain

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useful antitumor polysaccharides (*10–12*). Maitake has been frequently used to treat spleen and stomach ailments, to calm the nerves and the mind, and to treat hemorrhoids. Dry maitake has been used in the production of health foods, including teas, whole powders, powders of hot water extracts, granules, and drinks (*13*).

Many investigators have tried to cultivate mushrooms on solid artificial media for fruiting body formation in order to obtain bioactive polysaccharides (*14–18*). However, this method does not guarantee a standardized product, since the polysaccharide composition varies from batch to batch. As a result, recent attention has been paid to the use of submerged culture of higher fungi for the production of mycelial biomass and bioactive products, particularly extracellular polysaccharides (EPS) (*19–25*).

Although several investigators have reported antitumor activities for different polysaccharide extracts obtained from the fruiting bodies of *G. frondosa*, the biological activities of EPS prepared from submerged cultures of this fungus have received much less attention (*26–33*). Even so, these EPS have been shown to have several physiologically important activities (*9,10*). However, studies have focused mainly on medicinal uses, such as antitumor activity and immunostimulation, while relatively little work has been done to investigate the application of mushroom polysaccharides in cosmetics. There are two main fields of potential application in this area, namely as antioxidants to combat the effects of reactive oxygen species and as stimulators of tissue repair after damage, especially UV damage.

Reactive oxygen species (ROS), such as hydroxyl and superoxide radicals, are produced by sunlight, ultraviolet radiation, chemical reactions and metabolic processes. They have a wide variety of pathological effects on cellular processes (*34*). 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 (*35,36*). Free radicals are molecules or parts of molecules with unpaired reactive electrons, and can be generated by the metabolic processing of oxygen. They are hostile and damaging to cells and their functions. They can also initiate a chain reaction, with the multiplication of new free radicals. The damage that they cause includes tissue loosening, genetic damage and the promotion of disease and aging (*37*). Free radicals are implicated in many diseases such as heart disease, cancer, arthritis, and the aging process itself. To combat these free radicals the body needs antioxidants (*35*). The antioxidant properties of four specialty mushrooms, including maitake, were studied by Mau *et al.* (*38*). Almost all extracts of the fruiting bodies that they examined showed antioxidant activity; the maitake extract showed about 40 % free radical inhibition activity.

Ultraviolet (UV) irradiation damages human skin and causes premature skin aging (photoaging) through the activation of matrix metalloproteinases (MMPs), which are responsible for the degradation of collagen, gelatin and other components of the extracellular matrix (ECM) (*39*). Several investigators elucidated the influence of UVA irradiation on the stimulation of interstitial collagenase and gelatinase mRNA and their corresponding proteins in cultured human dermal fibroblasts (HDF) (*40,41*). Some types of polysaccharides have been suggested to play a role during the early stages of healing of a variety of connective tissues, for example in cell proliferation and in the synthesis of matrix components (*42,43*). Hyaluronan (HA) is a representative biomaterial for this use (*44*). The importance of HA has been extensively described for the homeostasis of connective tissues during embryogenesis and aging and its role in tissue repair. Croce *et al.* (*44*) elucidated the effect of exogenous HA on the synthesis of total protein and collagen in HDF. There were strong indications 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 (*44*). For this reason, HA has been widely used as a cosmetic ingredient for a long time. Other types of polysaccharides have been used as alternative ingredients for enhancing collagen biosynthesis in skin cells (*45,46*). EPS from *G. frondosa* may also have a potential for this application.

In our search for functional cosmetic ingredients from mushrooms, our group has produced several kinds of EPS in submerged culture of *G. frondosa* and investigated their biological activities, including antioxidative and free radical scavenging activities, as well as their ability to stimulate the proliferation of human dermal fibroblasts and also the biosynthesis of collagen in these cells. In this paper, we describe the production, characterization, biological activities, and application of EPS and MPS produced from submerged mycelial culture of *G. frondosa.*

Materials and Methods

Microorganism and media

G. frondosa HB0071 was maintained on potato dextrose agar (PDA) slants. Unless otherwise specified, slants were incubated at 27 °C for 5 days and then stored at 4 °C. The seed culture was grown in a 250-mL flask containing 50 mL of glucose medium. This medium contained (in g/L): glucose 30, yeast extract 6, polypeptone 2, $MgSO_4 \cdot 7H_2O$ 0.5, K_2HPO_4 0.5, and $MnSO_4 \cdot 5H_2O$ 0.2. It was incubated at 27 °C on a rotary shaker at 120 rpm for 5 days.

Submerged mycelial cultures for EPS production

The other seed cultures were performed in a 500-mL flask containing 100 mL of the media after inoculating with 3 % (by volume) of the seed culture, and then cultivated at 25 °C in a 5-litre stirred-tank bioreactor (KoBioTech Co., Incheon, Korea) or in a 5-litre airlift fermentor (Best Korea Co. Ltd., Daejeon, Korea). Fermentations were conducted at 25 °C, with an aeration rate of 1.16 vvm, an agitation speed of 166 rpm, a pH of 5.06, and a working volume of 3 L.

Preparation of EPS

The fermentation broth was centrifuged at 8000 × *g* for 20 min, and the resulting supernatant was filtered through Whatman filter paper No. 2 (Whatman International Ltd., Maidstone, UK). The filtrate was mixed with

four volumes of absolute ethanol, stirred vigorously and left overnight at 4 °C. The precipitated EPS was collected by centrifugation at 8000 × *g* for 10 min. The supernatant was discarded. The residue was reprecipitated with four volumes of ethanol and the precipitate of purified EPS was freeze-dried in a lyophilizer.

Preparation of mycelial polysaccharides

For isolation of mycelial polysaccharides, mycelial cells were obtained by filtration of culture broths. They were washed 3 times with distilled water then suspended in distilled water. The solutions were heated for 6 h in an autoclave at 121 °C to extract heat-stable mycelial polysaccharides, followed by filtration. The filtrates were then put through a process of ethanol precipitation, dialysis, and then the mycelial polysaccharide preparations were freeze-dried in a lyophilizer.

Estimation of mycelial growth and EPS production

Mycelial growth was estimated by the dry mass of the mycelium pellets after repeated washing with distilled water and drying overnight at 70 °C to a constant mass. EPS production was estimated by the dry mass of the lyophilized EPS precipitate. To measure the residual glucose, the filtrate was analyzed by high performance liquid chromatography (HPLC) using a Sugar-Pak column (300×6.5 mm, Waters Co., Milford, MA, USA) equipped with an evaporative light-scattering detector (ELSD, Alltech Associates, Deefield, IL, USA).

Evaluation of the mycelial morphological and rheological properties

The morphological properties of the mycelia were evaluated using an image analyzer (WINA Tech Co., Ansan, Korea) with software coupled to a light microscope through a charged coupled device (CCD) camera (Toshiba Co., Japan). The samples were fixed with an equal volume of fixative (prepared by mixing 13 mL of 40 % formaldehyde and 5 mL of glacial acetic acid into 200 mL of 50 % ethanol). Each fixed sample (0.1 mL) was transferred to a slide, air dried and stained with methylene blue (0.3 g of methylene blue, 30 mL of 95 % ethanol in 100 mL of water) (*47*). Rheological measurements using Brookfield programmable LVDVII+ digital viscometer (Brookfield Engineering Laboratories Inc., Stoughton, MA, USA) fitted with a small sample adapter were also performed on samples collected from the bioreactor at regular intervals.

EPS analysis of carbohydrates and amino acids

The total sugar content of EPS was determined by the phenol-sulphuric acid method using glucose as the standard (*48*). The sugar composition was analyzed by HPLC (Waters 2695 Separations Module, Waters Co., Milford, MA, USA) with a Sugar-Pak 1 column and an ELSD detector. The total protein was determined by the Lowry method with bovine serum albumin as the standard (*49*). The composition of amino acids was determined by HPLC with an AccQ·Tag Amino Acid Analysis column (150×3.9 mm, Waters Co., Milford, MA, USA) and a photoarray detector (996, Waters Co., Milford, MA, USA).

EPS molecular mass determination

The molecular mass of EPS was estimated on the basis of the calibration curve made by the HPLC system with a Shodex OHpak KB-804 column (300×0.8 mm, 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 ELSD detector. The column was standardized with dextrans of diverse molecular masses (Polymer Standards Service Inc., Silver Spring, MD, USA).

Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity was measured using the 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 (*50*) by measuring percent inhibition of NBT reduced by SOD. A volume of 1 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 on the plate. After adding 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.

Culture of human dermal fibroblasts (HDF)

HDF, isolated from human neonatal foreskin, were purchased from Modern Tissue Technologies Inc. (Seoul, Korea). They were cultured on Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture (DMEM/F-12; 3:1 by volume, Sigma) at 37 °C in a humidified atmosphere containing 5 % CO_2, 10 % fetal bovine serum (FBS), penicillin (100 IU/mL), and streptomycin (100 μg/mL). Fibroblast cultures were subcultured by trypsinization and used between the sixth and tenth passages.

UVA irradiation

HDF (1.5×10^5/well) were seeded into 35-mm diameter plates (CORNING®, Corning Inc., NY, USA) and cultured overnight. Prior to irradiation, when cells were 70–80 % confluent, they were washed twice with phosphate buffered saline (PBS, HyClone Laboratories, Logan, UT, USA). A UVA simulator (Jhonsam Inc., Seoul, Korea), filtered for the emission of UVA (320–400 nm), was used at a tube-to-target distance of 15 cm. The dose of UVA radiation, determined with a UV radiometer (International Light Inc., Newburyport, MA, USA) was set at 6.3 J/cm^2. After irradiation, fresh serum-free media containing EPS at different concentrations were added to cells at 37 °C for 24 h.

Measurement of free radical scavenging activity

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 cells were added to each well of a 24-well microtiter plate. The cells were maintained in a humidified atmosphere of 5 % CO_2 and 95 % air, at

37 °C. Analyses of confluent fibroblast cultures were carried out at 6–10 passages of subcultivation. 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). The UVA irradiation dose was 360 mJ/cm^2. A non-fluorescent compound, 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA), was able to react with free radical compounds, especially with hydrogen and to generate a fluorescent product. Cells were loaded with 5 μM DCF and 2 % Pluronic F-127 in HCSS solution (120 mM NaCl, 5 mM KCl, 1.6 mM $MgCl_2$, 2.3 mM $CaCl_2$, 15 mM glucose, 20 mM HEPES and 10 mM NaOH), incubated for 20 min at 37 °C and then washed three times with HCSS solution. The fluorescence signal of DCF (λ_{ex}=490 nm; λ_{em}=510 nm), the oxidation product of DCF-DA by free radicals, was then analyzed by flow cytometry (Becton Dickinson, NJ, USA) (*51*). Experiments were performed in triplicate.

Activity on the proliferation of fibroblasts

Mouse fibroblasts NIH 3T3 were cultured on DMEM containing 10 % FBS and then 2×10^5 cells were added to each well of a 24-well microtiter plate. After addition of 10 % (by mass per volume) of polysaccharide solution into each well, the 24-well plate was maintained at 37 °C in a CO_2 incubator for 2 days. After the cultivation was completed and DMEM removed, 60 μL of 0.5 % MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and 500 μL of fresh DMEM were added to each well. The plate was maintained in a CO_2 incubator for 2 h to allow 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 a microplate reader (Multiskan Ex, Thermo Electron Co., Finland). The proliferation of fibroblasts was evaluated by comparing the absorbance with that of the untreated control.

Activity on collagen biosynthesis

Collagen from fibroblasts was quantified by the Sirius Red method (*52*). Human dermal primary fibroblast p16 was seeded in 1-mL aliquots, in DMEM containing 10 % FBS, to give a density of 1×10^5 cells per well in a 24-well plate. After a 24-hour incubation at 37 °C, the medium was replaced with 1 mL of DMEM containing 10 % (by mass per volume) of polysaccharide solution. After 48 h, the cell culture supernatant and the cell extracts obtained by rapid freezing and thawing were dried onto the plate. The plates were incubated at 37 °C overnight (humidified) and then kept for 24 h at 37 °C. Each well was filled with 1 mL of 0.1 % (by mass per volume) 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, each wash lasting 10 s. 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 a microplate reader. Experiments were performed in triplicate.

RNA isolation and RT-PCR

RNA was extracted using an RNeasy Mini Kit (Qiagen, Maryland, USA) according to the supplier's instructions. First, a reverse-transcriptase polymerase chain reaction (RT-PCR) was performed to synthesize cDNA using an Omniscript RT Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR was then performed with each cDNA of the matrix metalloproteinase 1 (MMP-1), β-actin fragments, primers, and Taq DNA polymerase. The primers used were as follows:

MMP-1
5'-AAAGGGAATAAGTACTGGGC-3' (sense)
5'-AATTCCAGGAAAGTCATGTG-3' (anti-sense)
β-actin
5'-ATGCAGAAGGAGATCACTGC-3' (sense)
5'-CTGCGCAAGTTAGGTTTTGT-3' (anti-sense).

The primer sets yielded PCR products of 237 and 248 bp for MMP-1 and β-actin, respectively. Reactions were carried out in an automatic heat-block DNA thermal cycler (ASTEC PC801, ASTEC Inc, Tokyo, Japan) for 25 cycles: denaturation for 30 s at 94 °C; annealing for 30 s at 50 °C; extension for 60 s at 72 °C. PCR products were electrophoresed on a 1.5 % agarose gel in TAE (40 mM Tris acetate, 1 mM EDTA) and visualized by ethidium bromide staining. The level of expression of each mRNA gene was expressed as the ratio of the intensity of each gene PCR product to the corresponding β-actin PCR product (this being used as a reference molecule for measuring mRNA stability) and normalized to the control sample.

Cytotoxicity assay

The cell viability was determined by the modified method of Mosmann (*53*) using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, which involves the formation of a dark blue formazan product by mitochondrial dehydrogenase. HDF were cultured on DMEM/F-12 containing 10 % (by volume) FBS and then 2×10^4 cells were added to each well of a 96--well microtiter plate. After addition of EPS at different concentrations into the different wells, the 96-well plate was maintained at 37 °C for 24 h in a CO_2 incubator. After cultivation was completed and DMEM/F-12 removed, 12 μL of 0.5 % MTT and 100 μL of fresh DMEM were added to each well. The plate was placed in the CO_2 incubator for 4 h to allow formazan formation. The quantity of formazan produced can be regarded as an indicator of cell density or viability. After dissolving formazan in 100 μL of acid-isopropanol (0.04 M HCl in isopropanol), the absorbance at 570 nm was measured with a microplate reader (Model ELX 800, BIO-TEK Inc., Winooski, VT, USA). The results obtained were calculated from three sets of experiments and are presented as a percentage of control values.

Enzyme-linked immunosorbent assay (ELISA)

The expression level of MMP-1 was assayed by an enzyme-linked immunosorbent assay (ELISA). HDF (2×10^4

cells/well) were seeded into 48-well plates and cultured overnight. The culture media were replaced with DMEM/F-12 containing EPS at different concentrations. After 24-hour incubation, samples of the supernatants were transferred into a 96-well plate and a volume of coating buffer (Na_2CO_3 1.59 %, $NaHCO_3$ 2.93 %, NaN_3 0.20 %, $MgCl_2$ 1.02 %, pH=9.6) equal to the sample volume was added to each well. Plates were incubated for 24 h. The supernatants were removed and the coated well was washed three times with PBS containing 0.05 % Tween 20 (PBST) followed by blocking with 3 % bovine serum albumin in PBS for 1 h at 37 °C. After washing three times with PBST, 50 μL of 1/1000 diluted primary antibody (Ab), Ab-5 in PBST, were added into each well and incubated for 60 min. After washing the wells with PBST three times, 50 μL of 1/1000 diluted secondary Ab, anti-mouse IgG conjugated with alkaline phosphatase in PBST, were added and incubated for 60 min. After washing five times with PBST, 100 μL of 1mg/mL of *p*NPP (*p*-nitrophenylphosphate) in a diethanolamine buffer were added. The absorbance was measured at 405 nm after 30 min. Finally, cytotoxicity of the supplemented chemicals was measured by the MTT assay.

Inhibitory effect on melanin biosynthesis

First, the inhibitory effect of GF-glucans on melanin biosynthesis was investigated by measuring the inhibition of tyrosinase (a key enzyme in melanin biosynthesis) activity, as outlined by Kageyama *et al.* (*54*). Second, the melanin content was determined using a combined method outlined by Oka *et al.* (*55*) and Kageyama *et al.* (*54*). Mouse B16 melanoma cells were cultured in Eagle's minimal essential medium containing 10 % FBS, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 250 ng/mL of fungizone in a humidified atmosphere containing 5 % CO_2 at 37 °C. The cells from a subconfluent monolayer in a 10-cm culture dish were solubilized in 150 μL of 1 M NaOH, incubated at 80 °C for 2 h, and vortexed to solubilize the melanin. The absorbance of the solution was measured at 400 nm and was compared with a standard curve obtained using known concentrations of synthetic melanin (Sigma). The melanin content was expressed as μg/mg of protein.

Statistical analysis

All experiments were performed in triplicate. Data are presented as mean ± standard error (SE). Experimental results were analyzed statistically using Student's t-test (SigmaPlot 2000). P values less than 0.05 were considered to be statistically significant.

Results and Discussion

Submerged culture of Grifola frondosa

The fruiting body, mycelial formation on an agar plate, and typical mycelial morphology of *G. frondosa* in a stirred-tank bioreactor are presented in Fig. 1. One of the advantages of mycelial culture of *G. frondosa* is that it takes only 3.5 days to reach maximum mycelial biomass (24.8 g/L) and EPS production (7.2 g/L) in a stirred-tank bioreactor (Fig. 2). Note that many other macrofungi require up to 10 days to reach maximum mycelial biomass and EPS concentrations when grown in submerged cultures (*56,57*). *G. frondosa* therefore offers the potential for higher productivities of EPS in submerged culture. The optimum culture conditions for both mycelial biomass and EPS are presented in Table 1.

We investigated the growth and EPS production by *G. frondosa* in an airlift bioreactor since this type of bioreactor is often better for mycelial growth than stirred tank bioreactors, due to lower shear forces. However, the maximum concentrations of mycelial biomass (10 g/L) and EPS (4.53 g/L) obtained in the airlift bioreactor were lower than those in the stirred-tank bioreactor. It is probable that the stirred-tank bioreactor not only provided better mixing, but also promoted the formation of the desired morphology. Even when growth conditions are quite similar, the mycelium grows as feather-like mycelial clumps in the stirred tank bioreactor and as compact pellets in the airlift bioreactor (*27*).

In fact, our previous study of the morphology of growth in stirred tank bioreactors showed that *G. frondosa* mainly forms pellets with high hairiness, with pellet size increasing rapidly from the beginning of the fermentation and reaching a maximum value at day 4, which corresponded to the time of maximum biomass and EPS concentrations (*27*). After this period, the core region became denser and larger, due to a lack of nutri-

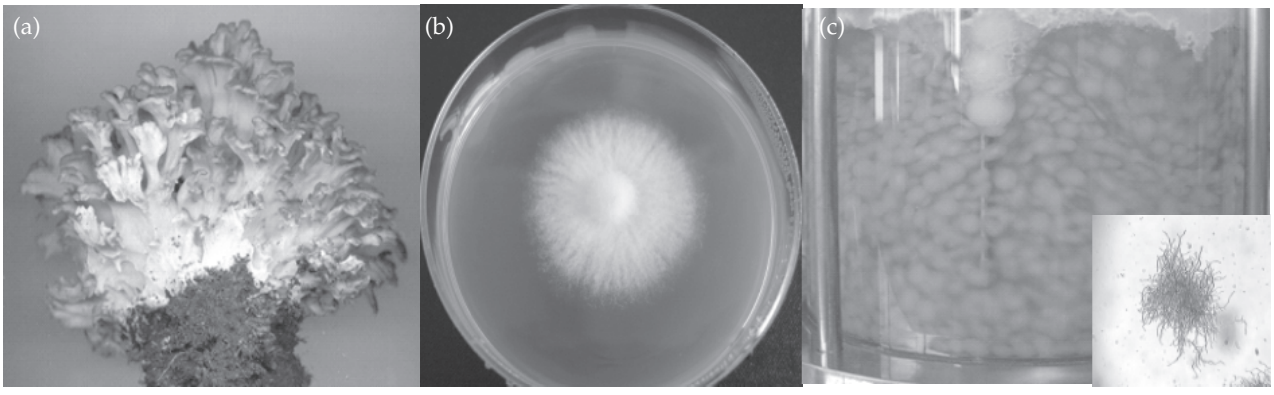

Fig. 1. Fruiting body (a), mycelial growth (b) on PDA medium (at day 3), and mycelial pellet growth (c) in a stirred-tank bioreactor (at day 6) of *Grifola frondosa* HB0071

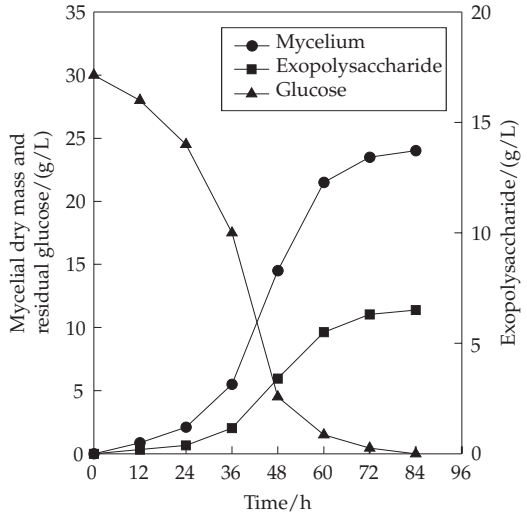

Fig. 2. Typical time profiles of mycelial biomass and exopolysaccharide production in submerged culture of *Grifola frondosa* in a 5-litre stirred-tank bioreactor, under optimized conditions (controlled pH=5.06, aeration volume 1.16 vvm, agitation speed 166 rpm)

Table 1. Optimum culture conditions of *Grifola frondosa* for the production of mycelial biomass and EPS

Items	Condition
Carbon source	Glucose, 30 g/L
Nitrogen source	Yeast extract, 6 g/L
Inoculum size	3 % (by volume)
pH and temperature	pH=5.06* and 25 °C
Aeration rate	1.16 vvm*
Agitation intensity	166 rpm*

*Conditions optimized by self-directing optimization method

ent and oxygen supply. Later, the larger pellets broke into several smaller pellets, but without significant hyphal fragmentation, since there was no significant increase in the concentration of free mycelia. The culture pH, aeration rate, and hydrodynamic behavior affected the growth morphology and the changes during the culture: compact pellets were formed under low aeration, whereas freely suspended mycelial growth was obtained under high aeration. Maximum mycelial biomass and polysaccharide levels were achieved when the biomass grew as loose mycelial clumps with high hairiness.

Our observations about the morphology of growth in our previous study prompted us to investigate the rheology of the EPS in the present work. Fig. 3 shows that an aqueous solution of GF-glucan demonstrated a pseudoplastic flow behavior, and the viscosity of GF-EPS was similar to mannans and higher than hyaluronic acid.

Characterization of GF polysaccharides

Five polysaccharide preparations were characterized, namely a mycelial extract (GF-MPS) and the top and bottom fractions of filtrate precipitates obtained after the

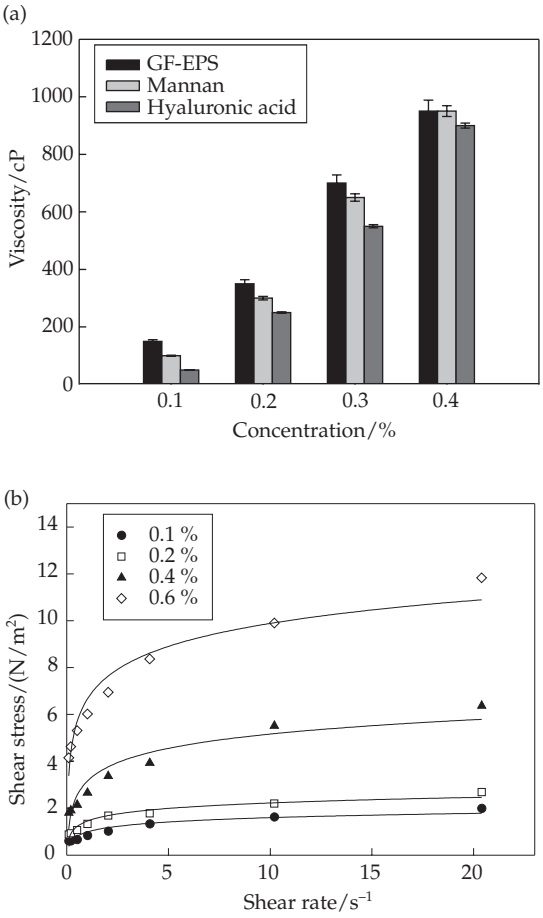

Fig. 3. Viscosity (a) and shear rate versus shear stress (b) of the GF-EPS solution

submerged culture of *G. frondosa* in two different media (glucose medium and PMP medium, the latter consisting of potato dextrose broth 24 g/L, malt extract 10 g/L, and peptone 1 g/L). The EPS preparations had diverse molecular masses, ranging from 470 to 1650 kDa, and their individual biological activities were different (*8*). These EPS preparations contained 66–94 % polysaccharide and 6–34 % protein. The protein moiety of the GF--EPS contained 16 kinds of amino acids, the main ones being threonine, alanine, valine and glutamine; the carbohydrate moiety contained four sugars, the main ones being glucose and galactose.

Cytotoxicity of GF polysaccharides

Cytotoxicity was evaluated by measuring the mitochondrial dehydrogenase activity in cells. This assay is based on the active transport of MTT (a tetrazolium salt) into the cell, which is then reduced to a formazan byproduct by mitochondrial dehydrogenases (*53*). This test was used to investigate the effect of different doses of EPS on the viability of human dermal fibroblasts. Cells were incubated with EPS concentrations of 1–4 % for 24 h. In all groups, the EPS did not significantly affect cell viability nor did it cause morphological changes (Fig. 4). In fact, the cell viabilities were higher after the treatments with either GF-EPS or GF-MPS than they were with β-glucan and sodium hyaluronate, compounds that

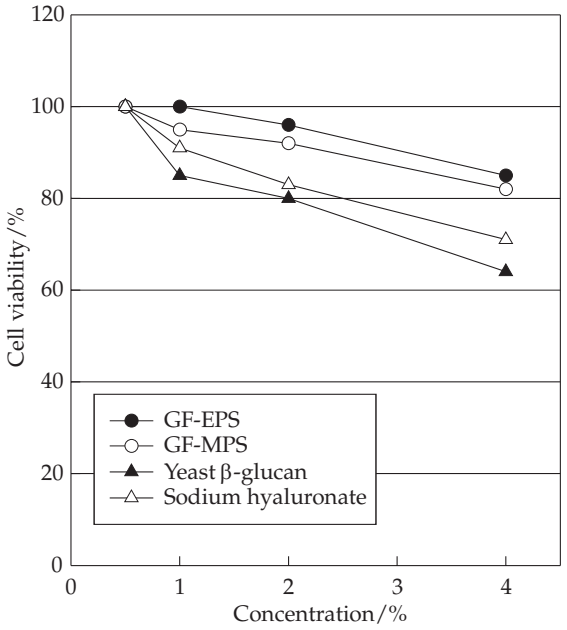

Fig. 4. The cytotoxicity of GF-EPS and GF-MPS produced by submerged culture of *G. frondosa* HB0071 with reference to yeast β-glucan and sodium hyaluronate. The cell viability is expressed as a percentage of viable cells in comparison with the viable cells in the reference compounds. The cytotoxicity is significant ($p<0.05$) and the values are mean ± S.E.

have been widely used as cosmeceuticals. Therefore, within the dosage range tested, human dermal fibroblasts treated with EPS did not exhibit any cytotoxicity.

Antioxidant activity of GF polysaccharides

The antioxidant activities of the GF-EPS and GF-MPS were investigated by measuring the superoxide scavenging activity. As shown in Fig. 5, both GF-EPS and GF-MPS had high antioxidant activity, in a dose-dependent manner. The GF-MPS showed higher activity (89 % at 0.2 %) than that of GF-EPS (50 % at 0.2 %). However,

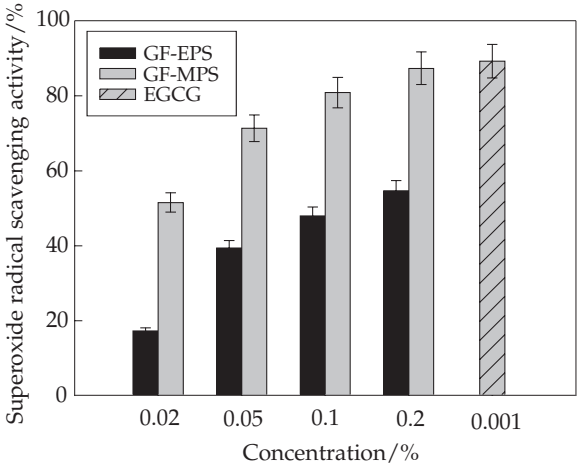

Fig. 5. Superoxide radical scavenging activity of GF-EPS and GF-MPS produced by submerged culture of *G. frondosa* HB0071. EGCG was used as a positive control. The activity is expressed as a percentage increase in comparison with that of control. The activity is significant ($p<0.05$) and the values are mean ± S.E.

this activity was lower than (-)-epigallocatechin-3-gallate (EGCG), a main component of green tea extract (89 % at 0.001 %). Antioxidative activity of mushroom polysaccharides has been found in other species (*58–61*).

Free radical scavenging activity of GF polysaccharides after UV irradiation

At a concentration of 0.2 % (by mass per volume), both GF-EPS and GF-MPS decreased free radical levels (formed after UV irradiation) by approximately 20 % (Fig. 6). However, this is a relatively low free radical scavenging activity when compared to the activities of green-tea extract and vitamin C, which are potent free radical scavengers (*8*). Liu *et al.* (*62*) studied the free radical scavenging activity of various mushroom polysaccharides of diverse forms (*e.g.* mycelium extract, fruiting body extract, and culture filtrate). Five mushroom polysaccharide extracts and a protein-bound polysaccharide exhibited significant superoxide and hydroxyl radical scavenging activities. Kim and Kim (*63*) reported that a hot-water extract of the fruiting body of *Ganoderma lucidum* provided radioprotection and antioxidant defense against oxygen radical-mediated damage after metal-catalyzed Fenton reactions and UV irradiation. In fact, a water-soluble polysaccharide, isolated from the fruiting 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 (*63*).

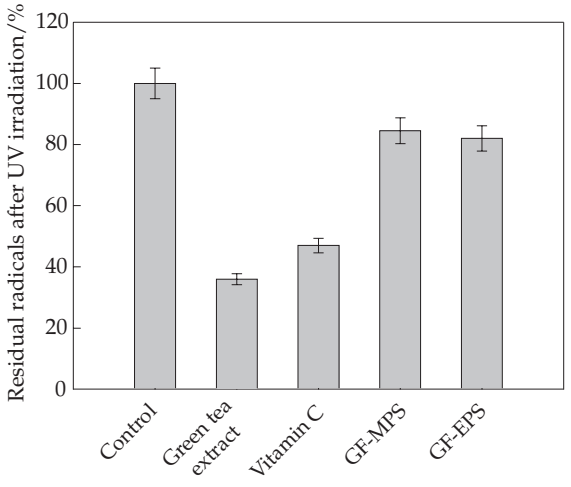

Fig. 6. Free radical scavenging by GF-EPS and GF-MPS in the UV irradiated human fibroblast. The activity is significant ($p<0.05$) and the values are mean ± S.E.

Effect of GF polysaccharides on UVA-mediated MMP-1 expression

In the present study, in order to estimate the effect of EPS on matrix metalloproteinase 1 (MMP-1) expression in UVA-irradiated HDF (6.3 J/cm^2), the ELISA method was used to quantify MMP-1 in the culture medium of HDF. The treatment of UVA-irradiated HDF with both GF-EPS and GF-MPS, both at concentrations of 0.2 % (by mass per volume), decreased the expression

of MMP-1 by 20 and 40 %, respectively (Fig. 7). Surprisingly, the inhibitory effect at an EPS concentration of 100 μg/mL was significantly higher than that of *trans*-retinoic acid (*t*RA), which is widely known as an inhibitor

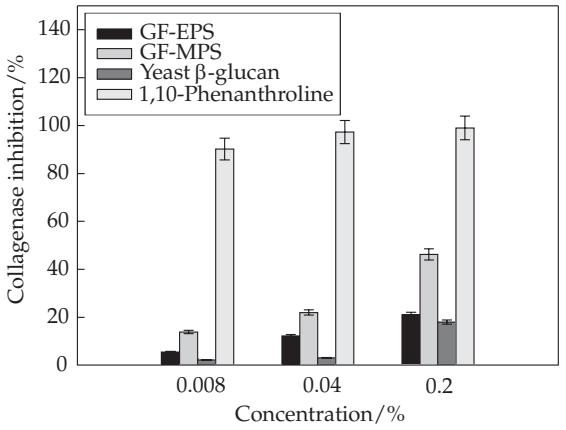

Fig. 7. MMP-1 (collagenase) inhibitory activity of GF-EPS and GF-MPS produced by submerged culture of *G. frondosa* HB0071. Yeast β-glucan and 1,10-phenanthroline were used as positive controls. The activity is expressed as a percentage increase in comparison with that of control. The activity is significant ($p<0.05$) and the values are mean ± S.E.

of UVA-induced MMPs (*9*). Fisher *et al.* (*64*) reported that *t*RA, applied to human skin, inhibits subsequent activation of activator protein (AP)1, a step that is essential for transcription of MMPs, and the induction of MMPs by UVA irradiation. Several investigators have reported that microbial glucan stimulated macrophage release of wound growth factors, which modulated fibroblast collagen biosynthesis (*65,66*). Recently, Kougias *et al.* (*67*) have reported the presence of at least two glucan binding sites on normal human fibroblasts.

Effect of GF polysaccharides on cell proliferation activity

The capability of GF-EPS and GF-MPS to stimulate proliferation of skin cells was investigated at concentrations of 0.05–0.2 % (by mass per volume). As shown in Fig. 8, both GF-EPS and GF-MPS stimulated the proliferation of fibroblasts by approximately 21–25 %, in a dose-dependent manner. Their activities were higher than that of β-glucan, but lower than that of epidermal growth factor (EGF). Other useful materials, such as biscoclaurine alkaloid cepharanthine and sodium lauryl sulfate (SLS), have been reported for fibroblast proliferation (*68–70*).

Collagen biosynthesis stimulating activity of GF polysaccharides

In the present study, the treatment of fibroblasts with GF-MPS, at a concentration of 0.2 % (by mass per volume), stimulated an increase of 53 % in the biosynthesis of collagen, while GF-EPS and yeast β–glucan caused lower stimulation levels (Fig. 9). Fig. 10 shows the effect of GF-EPS on the expression of type I collagen mRNA in human dermal fibroblasts. Fibroblasts were cultured with different doses of GF-EPS for 12 h and their expression

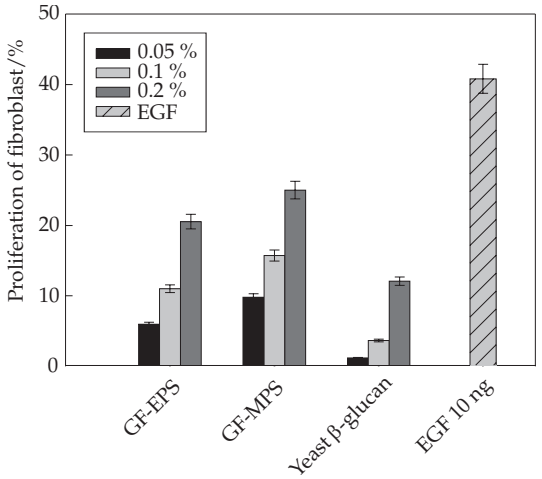

Fig. 8. The stimulation of cell proliferation by GF-EPS and GF-MPS produced by submerged culture of *G. frondosa* HB0071. Yeast β-glucan and EGF (epidermal growth factor) were used as positive controls

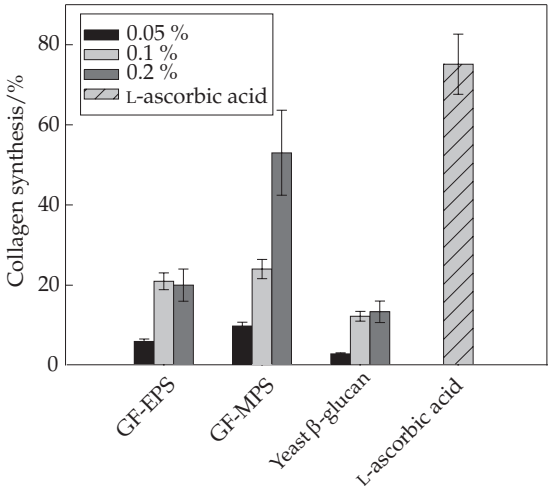

Fig. 9. The stimulation of type I collagen biosynthesis with GF-EPS and GF-MPS produced by submerged culture of *G. frondosa* HB0071. Yeast β-glucan and L-ascorbic acid were used as positive controls. The activity is significant ($p<0.05$) and the values are mean ± S.E.

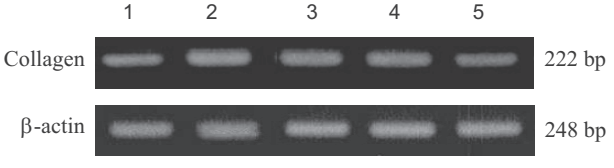

Fig. 10. Effect of GF-EPS on the expression of type I collagen mRNA in human dermal fibroblast. Fibroblasts were cultured with varying doses of GF-EPS for 12 h and compared with the control. Total RNA extracted from human dermal fibroblast was analyzed by RT-PCR. 1) Control, 2) 200 μM ascorbic acid, 3) 0.2 % GF-EPS, 4) 0.1 % GF-EPS, 5) 0.05 % GF-EPS

levels were compared with the control. GF-EPS treatment of cells led to increased mRNA levels, although the levels were lower than those in cells treated with ascorbic acid.

Inhibitory effect of GF polysaccharides on melanin biosynthesis

Tyrosinase controls the first and the rate-limiting step of melanogenesis, and is therefore the key enzyme of this cascade. Consequently, it is the target of different effectors that regulate melanin synthesis. In this study, GF--MPS inhibited the tyrosinase activity by 30 % at a concentration of 0.04 %, whereas GF-EPS showed no significant effect. The positive control, kojic acid, caused 50 % inhibition at a concentration of 0.04 % and the effect was dose-dependent (Fig. 11). Treatment of B16 mouse melanoma cells with GF-EPS and GF-MPS resulted in the inhibition of melanogenesis by 25 and 17 %, respectively (Fig. 12). These results imply that the inhibitory effect of GF-EPS on melanogenesis does not result from a direct inhibition of tyrosinase activity but rather from a more complicated phenomenon mediated by cellular signaling events. The inhibitory effect of GF-EPS (0.2 % treatment) was similar to arbutin. Arbutin is a popular active substance, originating from natural plants, which can whiten and lighten the skin. It can infiltrate into the skin quickly without affecting cell multiplication and effectively prevent the activity of tyrosinase in the skin. Consequently, it also prevents the formation of melanin (*71,72*).

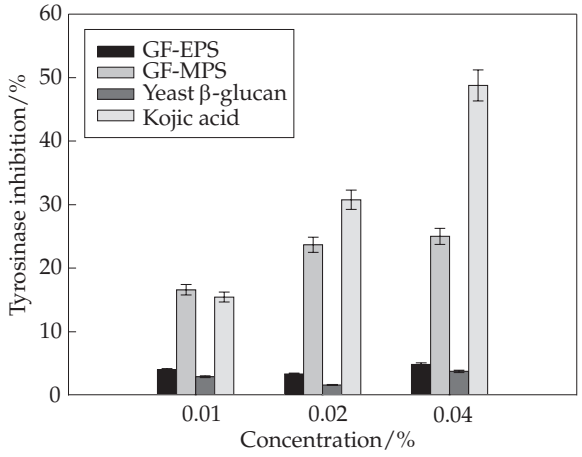

Fig. 11. Tyrosinase inhibitory activity of GF-EPS and GF-MPS produced by submerged culture of *G. frondosa* HB0071. Yeast β-glucan and kojic acid were used as positive controls. The activity is expressed as a percentage inhibition in comparison with that caused by the control. The activity is significant ($p<0.05$) and the values are mean ± S.E.

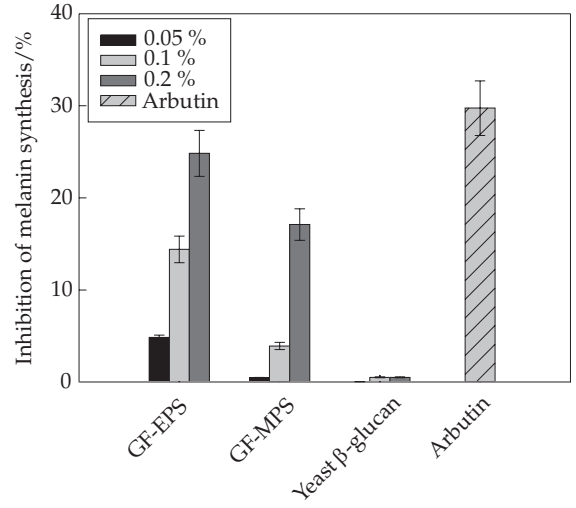

Fig. 12. Inhibition of melanin synthesis by GF-EPS and GF-MPS in B16 melanoma. Yeast β-glucan and arbutin were used as positive controls. The activity is significant ($p<0.01$) and the values are mean ± S.E.

Conclusion

Our results suggest that GF polysaccharides obtained from a mycelial culture broth of the new isolate of *G. frondosa* are a potential candidate to reduce MMP activity in the skin after solar stimulation. In the future, combinations of other natural compounds may be envisaged for more efficient photoprotection and a further study for elucidating overall biological functions of the polysaccharides *in vivo* should be performed.

Acknowledgement

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