New Anti-aging & Moisturizer Ingredients of Exopolysaccharides by *Grifola frondosa*

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ABSTRACTS

In this study, in an attempt to search for functional cosmetic ingredients from higher fungal, we have produced exopolysaccharides (GF-1, approximately carbohydrate 75%, protein 25%) and polysaccharide (GF-2) of mycelium extract, by submerged culture of *Grifola frondosa.*

For applications in anti-aging cosmetic field, we investigated the diverse biological activities.

Antioxidant activity and inhibition of Matrixmetalloproteinases (MMPs) were investigated enzymatic assays by measuring the superoxide scavenging activity using xanthine-xanthine oxidase system and the proteolytic activity of MMPs using EnzChek Collagenase/Gelatinase kits, respectively. GF-1 polysacchairde showed inhibition of superoxide radical by 90% at a concentration of 0.2% (w/v) and inhibition of collagenase by 45% at 0.2% (w/v). GF-2 polysaccharide of mycelium extract also exhibited good antioxidant activity. However, MMPs inhibition activity was relatively lower level compared to GF-1 polysaccharides.

The treatment of human dermal fibroblast (HDF) with GF-1 and GF-2 polysaccharides increased the proliferation of fibroblast by approximately 23~25% at a concentration of 0.5% (w/v), also showed collagen synthesis increase in HDF by about 50% at 0.5% (w/v) compared to that of untreated control.

We also report the moisturizing effects of polysaccharides in cosmetic products (O/W emulation) and its own ingredient, *in vitro* and *in vivo.* The GF-1 polysaccharide showed higher moisturizing ability than sodium hyaluronate, which is the most commonly used moisturizers ingredient.

These results suggest the GF-1 polysaccharide, protein-bound polysaccharide, may be used as an ingredient for new moisturizing and anti-aging cosmeceuticals.

INTRODUCTION

During the past decades, much interest has been generated in polysaccharides produced by numerous microorganisms specially mushrooms because of their various biological and pharmacological activities. Recent studies are carried out searching for new active agents to benefit the skin health and beauty industries.

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-) [1-3].

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 [4]. 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 [6]. 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 regarded as a good candidate for blocking the skin aging process. 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 [7-10].

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 [11]. Also, 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 [12]. Mau et al. [13] 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. 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. 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 [14,15]. Recently, several polysaccharides have been used as alternative ingredients for enhancing collagen biosynthesis and increasing

cell proliferation in the skin cells [16, 17]. Therefore, polysacchrides induce the production of extracellular matrix (ECM) such as collagen. The extracellular matrix (ECM) serves not only as a scaffolding to stabilize tissue structure, but also has been observed to influence the development, migration, proliferation, shape and metabolic function of cells that contact it. Matrix metalloproteinase (MMPs; e.g. gelatinases, collagenases and stromelysins), which digest collagen, gelatin and other components of the ECM, are important for both normal development and carcinogensis. Therefore, Matrix degradation is one of the important factors of wrinkle formation.

In this study, in an attempt to search for functional cosmetic ingredients from mushrooms, we have produced exopolysaccharides (GF-1) and polysaccharide (GF-2) of mycelium extract by submerged culture of *G. frondosa* and investigated their diverse biological activities including antioxidant activity, free radical scavenging activity after UV irradiation, inhibition of MMPs, proliferation of the fibroblasts, and collagen biosynthesis activity. Also, we have measured the moisturizing effects of polysaccharides to improve the skin condition.

To the best of our knowledge, this is the first report on the wide application of polysaccharides produced from submerged culture of mushrooms to new moisturizing and anti-aging cosmeceuticals.

MATERIALS AND METHODS

Microorganism and fermentation

A culture of *G. frondosa* was of our culture collection isolated from mountainous district in Korea. *G. frondosa* was initially grown on PDA medium in a petridish, and then transferred to the seed culture medium. The fermentation medium was inoculated with 3 % (v/v) of the seed culture and then cultivated at 25℃ in a 5-l stirred-tank fermenter (Best Korea, Daejeon, Korea).

Preparation of polysaccharides (GF-1 & GF-2 polysaccharide)

The fermentation broth was centrifuged 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℃. The fraction of polysaccharide in filtrate was designated exopolysaccharide (GF-1). To extract intracellular polysaccharides, the mycelial biomass was submerged in hot water for 4 h at 100℃ followed by extracted with ethanol for 24 h at 4℃, and the resulting polysaccharide was name as GF-2 polysaccharide.

Cosmetic preparations

For comparative skin hydration test, oil-in-water (O/W) emulsions were prepared with normal

base cream as a control and the same three base creams containing GF-1 (1.0 mg/ml) & GF-2 (1.0 mg/ml) polysaccharide and sodium hyaluronate (1.0 mg/ml) of 10% concentration, respectively.

Antioxidative 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 [12] by measuring percent inhibition of NBT reduction 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. The plates were incubated for 20 min at 37℃. After adding xanthine oxidase, the formazan produced was measured spectrophotometrically at 560 nm.

Measurement of free radical scavenging activity after UV irradiation

Human dermal fibroblasts (HDF) were cultured on plastic culture dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml). The cells were maintained in a humidified 5% CO_2-95% air incubator at 37℃. 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). UVB 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℃ and washed three times with HCSS solution, and the fluorescence signal of DCF (Ex=490 nm;Em=510 nm), the oxidation product of DCDHF-DA by free radicals, was analyzed flow by a cytometry (Becton Dickinson, NJ, USA). Experiments were performed in triplicate and their mean values were noted.

Matrixmetalloproteinase (MMP) Fluorometric assays

Fluorometric assays of the proteolytic activities of MMP-1 were performed using EnzChek Collagenase/Gelatinase kits. The assays were optimized to minimize the quantity of enzymes and substrates used. The enzymes were mixed with quenched fluorescent substrates (25ug/assay

of fluorescein-conjugated gelatin) in a final volume of 200ul of reaction buffer in 96-well microplates. The enzymatic assays were optimized with 0.1units of MMP-1 (provided in the EnzChek assay kit). The rate of proteolysis, in the presence or absence of inhibitor, was determined by measuring the increase in fluorescence using a fluorescence spectrophotometer (Perkin Elmer, USA.). For the MMP tested the activities under these conditions were linear for at least 15 min. Digested products from DQ gelatin (collagen) substrates have absorption maxima at ~ 495 nm and fluorescence emission maxima at ~ 515 nm.

Proliferation of fibroblasts

Human dermal fibroblasts (HDF) were cultured on Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS (fetal bovine serum) and then 2×10^5 cells/well were added on a 25-well micro-titer plate. After addition of polysaccharides into each well, the 24-well plate was maintained at CO_2 incubator (37℃) 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, 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:

$$[O.D_{565}(treated)-O.D_{565}(blank) / O.D_{565}(control)-O.D_{565}(blank)] \times 100$$

Collagen biosynthesis activity

Collagen from fibroblasts was quantified by the Sirius Red. Human dermal fibroblast (HDF) were seeded in 1ml 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℃ the medium was replaced with 1ml DMEM containing polysaccharides. After 48 h, cell culture supernatant and cell extract lysed by rapid freezing and thawing, was dried onto the plate. The plates were incubated at 37℃ for overnight (humidified) and then kept for 24 h at 37℃. 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 1h at room temperature. The plate was washed five times with 2 ml of 10 mM HCl for 10 sec/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.

Measurement of skin hydration and TEWL

Hydration testing on the skin was measured moisture content by Corneometer CM 825

(Courage-Khazaka Electronic, Co-logne, Germany). The corneometer measures changes of electrical capacity that are related to the moisture contents of the skin before and after applying the moisturizing products.

Transepidermal water loss (TEWL) after application of moisturizing products was measured using the Tewameter TM 210(Courage-Khazaka Electronic, Co-logne, Germany).

The left forearm of seven healthy volunteers was treated with each sample (1.0 mg/ml) and O/W emulsion containing 10% at the concentration of 0.1% (w/v) of each sample, respectively. The measurements were performed immediately after application at 10, 30, 60 and 120 minutes later. Untreated skin of the left forearm was used as a control. Volunteers were adapted to 31℃/40%RH for 45 minutes prior to the measurements in Room Temperature & Humidity Chamber. The recorded capacitance values were converted into arbitrary capacitance units (A.U.) varying from 0 – 120 A.U. and the measured value units of transepidermal water loss (TEWL) expressed in g/hm^2.

RESULTS AND DISCUSSION

Characterization of polysaccharides

The maximum mycelial biomass and polysaccharides produced were 17 g/l and 5.3 g/l, respectively at 13 days of fermentation using a 5-L stirred-tank bioreactor. The molecular mass and the ratios of carbohydrate to protein for each polysaccharide were investigated. The GF-1 polysaccharide (MW 770kDa) obtained from culture filtrates had higher molecular weight than GF-2 polysaccharide (MW 500kDa) of mycelium extracts. There was a difference in the ratios of carbohydrate to protein both of them (data not shown).

Antioxidative activity

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 [18, 19].

In the present study, anti-oxidant activity for GF-1 & GF-2 polysaccharides was investigated. As shown in Fig. 1, anti-oxidant activity of GF-1 polysaccharide was strongest among the polysaccharides and green-tea extract, which is widely known as potent anti-oxidant activity and free radical scavengers. GF-1 polysaccharide showed inhibition of superoxide radical by 90% at a concentration of 0.2% (w/v). GF-2 Polysaccharide from mycelium extract also exhibited good antioxidant activity.

Using these two polysaccharides, free radical scavenging activity after UVB irradiation in human dermal fibroblast cells was further studied *in vitro*. GF-1 and GF-2 polysaccharides

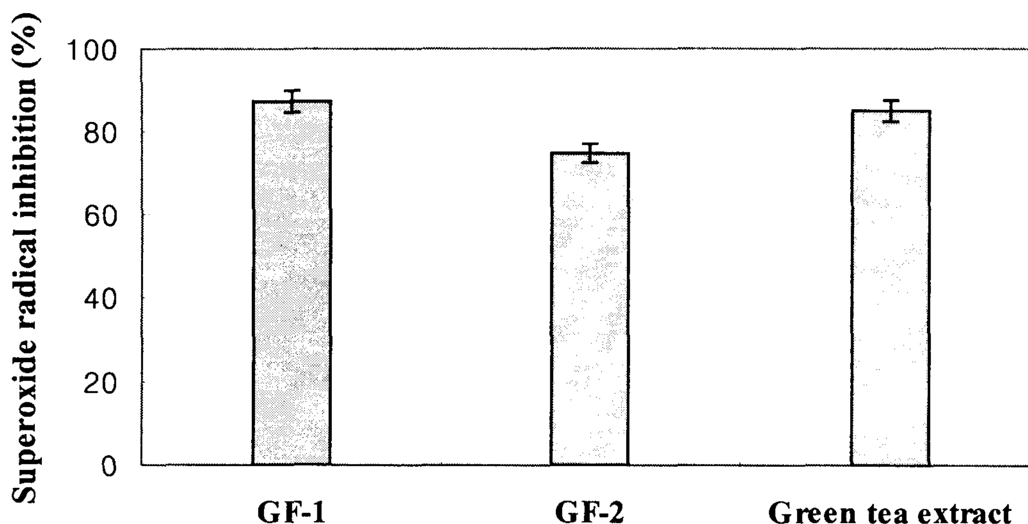


Figure 1. Comparison of antioxidant activity between two of groups of polysaccharides produced by submerged culture of *Grifola frondosa* and green-tea extract. The activity was measured by addition of 0.2% (w/v) polysaccharide and indicated by percentage of increase in comparison with that of control. The activity is significant ($P < 0.01$) and the values are mean±S.E.

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 (Fig. 2). Liu et al. [14] reported extensive results on free radical scavenging activity of various mushroom polysaccharides of diverse forms (e.g. mycelium extract, fruiting body 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.

Inhibition of MMP-1 (collagenase) activity

In this study, the inhibition of MMP-1 (collagenase) for GF-1 and GF-2 polysaccharide was investigated. To comparative study of inhibition of MMP-1, we have used to other type of polysaccharide, which are hyaluronic acid, pullulan and yeast-glucan. Collagenase purified from *Clostridium histolyticum* is provide with the EnzChek Gelatinase/Collagenase Assay Kit to serve as a control enzyme.

As shown in Fig. 3, GF-1 polysaccharide showed inhibition of collagenase by 45% at 0.2% (w/v). However, GF-2 polysaccharide from mycelium extract and other type polysaccharides were lower level compared to that of GF-1 polysaccharide. We confirmed that polysaccharide from *G. frondosa* showed the ability to active agent for skin anti-aging.

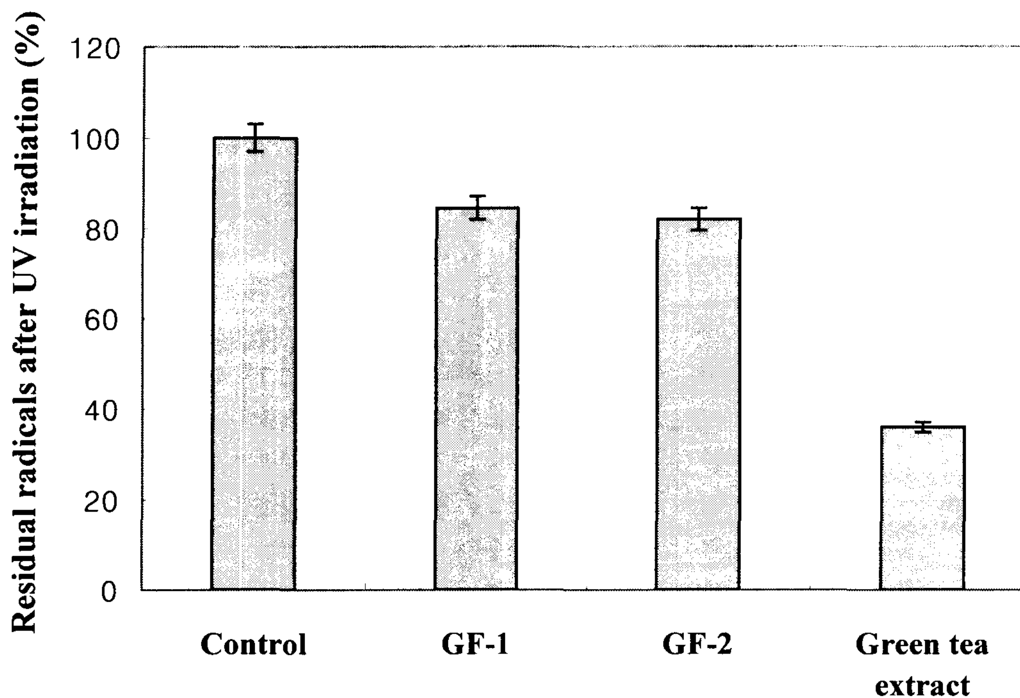


Figure 2. Comparison of free radical scavenging after UV irradiation between two of groups of polysaccharides produced by submerged culture of *Grifola frondosa* and green-tea extract. The activity was measured by addition of 0.2% (w/v) polysaccharide. The activity is significant ($P < 0.01$) and the values are mean±S.E.

Proliferation of fibroblasts

Several applications have been reported for useful materials to skin cell proliferation. Ohkura et al. [20] 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. Kim et al. [21] reported that β-glucan produced from *Schizophyllum commune* increased the proliferation of fibroblasts by 40% at a concentration of 0.04%.

In the present study, for GF-1 and GF-2 polysaccharides, the ability to affect proliferation of skin cells was investigated at a concentration of 0.5% (w/v). As shown in Fig. 4, the GF-1 and GF-2 polysaccharides increased the proliferation of fibroblasts by approximately 23~25%.

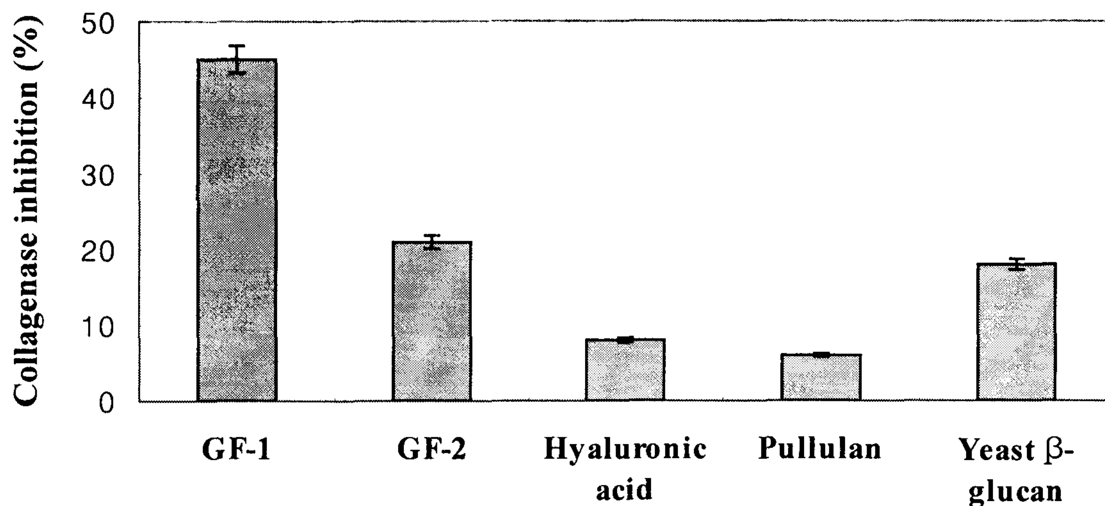


Figure 3. Comparison of MMP-1 inhibition activities GF-1 and GF-2 polysaccharides produced by submerged culture of *Grifola frondosa* and hyaluronic acid, pullulan and yeast-glucan. The activity was measured by addition of 0.2% (w/v) polysaccharide, The activity is significant ($P < 0.01$) and the values are mean±S.E.

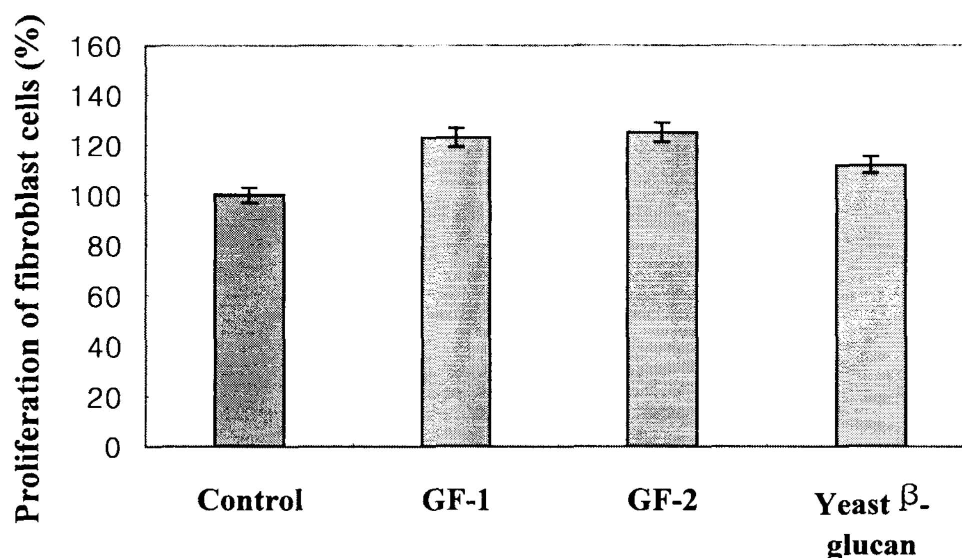


Figure 4. Comparison of proliferation of fibroblast between two of groups of polysaccharides produced by submerged culture of *Grifola frondosa* and yeast β-glucan. The activity was measured by addition of 0.5% (w/v) polysaccharide. The activity is significant ($P < 0.01$) and the values are mean±S.E.

Collagen biosynthesis activity

The major fibrillar protein of extracellular matrixs (ECM) is collagen. Collagens are important because they are responsible for tissue strength and elasticity and also because of their dynamic role in promoting cell growth and differentiation. Hyaluronan (HA), one of major ECM components, has been regarded as a representative biomaterial for this use [15]. The importance of HA has been extensively described 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 human dermal fibroblasts. Recently other types of polysaccharides have been used as alternative ingredients for enhancing collagen biosynthesis in skin cells. For example, treatment of β-glucan produced from *S. commune* increased collagen biosynthesis by 32% at a concentration of 0.04%, whereas yeast β-glucan (from *Saccharomyces cerevisae*) gave at most a 10% increase in collagen biosynthesis [21]. More recently, it has been reported that the anabolic steroid stanozolol enhanced collagen synthesis in a dose-dependent manner in cultures of adult human dermal fibroblasts [22]. The stimulatory effects of stanozolol on collagen synthesis were blocked by a TGF-beta1 anti-sense oligonucleotide, by antibodies to TGF-beta, and in dermal fibroblast cultures derived from TGF-beta-1 knockout mice.

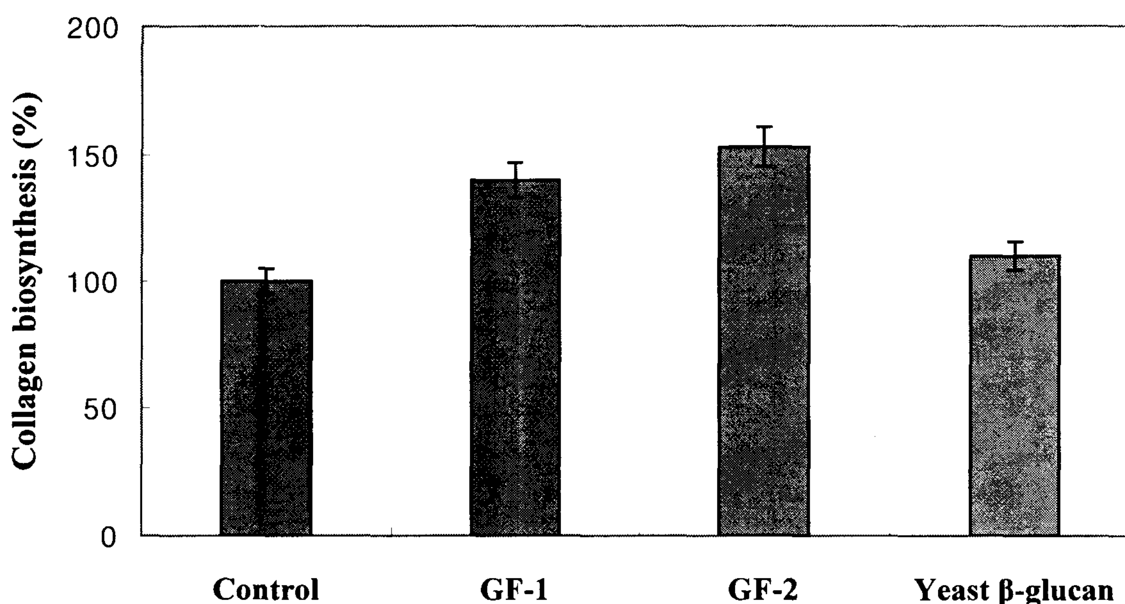


Figure 5. Comparison of collagen biosynthesis activities between two of groups of polysaccharides produced by submerged culture of *Grifola frondosa* and yeast β-glucan. The activity was measured by addition of 0.5% (w/v) polysaccharide. The activity is significant ($P < 0.01$) and the values are mean±S.E.

In this study, the treatment of fibroblasts with GF-1 and GF-2 polysaccharides increased in the biosynthesis of collagen by approximately 50% (Fig. 5). It means that polysaccharides showed inhibitory activity on collagenase activity (Fig. 3). Accordingly, polysaccharides from submerged culture of *G. frondosa* can be used as collagenase inhibitor agents for anti-aging cosmetic products.

Skin-Moisturizing effect of GF-1 & GF-2 polysaccharides

Figure 6 Shows the changes of electrical capacitance increase ratio with time after application as the moisturizer raw materials (ingredient) and cosmetic products (O/W emulsion) containing 10% at a concentration of 0.1% GF-1 & GF-2 polysaccharides and sodium hyaluronate measured at 31° ± 0.2℃ and 40% RH, respectively. The GF-1 polysaccharide showed higher moisture contents of the skin than those of GF-2 polysaccharide and sodium hyalunate, which is widely known as moisturizer ingredient, after 120 mins.

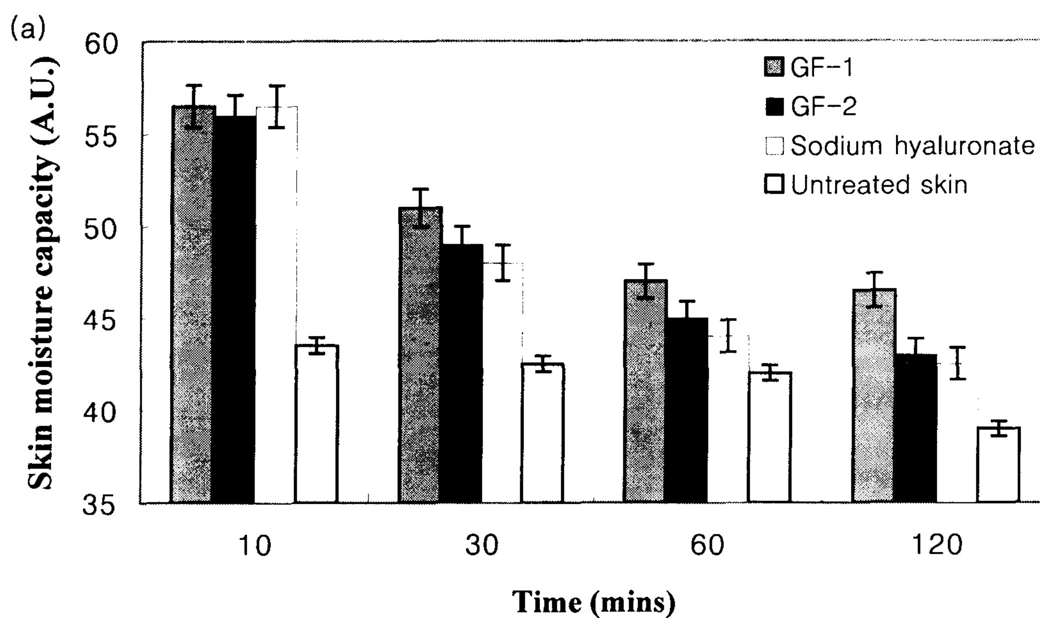
The transepidermal water loss (TEWL) of the skin is an important parameter for evaluating the efficiency function of the skin water barrier. As showed in Fig 7, the water loss content values of GF-1 polysaccharide were relatively a little decrease compared to those of GF-2 polysaccharide and sodium hyalunate. Accordingly, there may be due to the fact that the viscosity of GF-1 polysaccharide is higher than that of GF-2 polysaccharide or sodium hyalunate (data not shown). For this reason, GF-1 polysaccharide will be used for a important cosmetic ingredient to protect our skin from stressed condition or low humidity condition.

CONCLUSION

We show that polysacchairdes (GF-1 & GF-2 polysaccharide) from *Grifola frondosa*, are a new active component suitable for cosmetic applications. The biological activities of the polysaccharides have been studied, including moisturizing effects, for functional cosmetic ingredients uses from fungal, we have produced exopolysaccharides, GF-1 (approximately carbohydrate 75%, protein 25%) and polysaccharide, GF-2 of mycelium extract, by submerged culture of *G. frondosa*.

In the present study, GF-1 polysaccharide from fermentation broth of *G. frondosa* showed inhibition of superoxide radical and inhibition of MMP-1 (collagenases) by *in vitro* enzyme assay, respectively. In cell culture experiment, HDF pretreatment with GF-1 polysaccharide increased the proliferation of fibroblast and showed collagen synthesis increase in HDF compared to that of untreated control.

The pretreatment of skin with cosmetic formulations (O/W emulation) containing GF-1 polysaccharide showed relatively higher moisture contents and water loss contents in the skin compared to those of sodium hyaluronate.



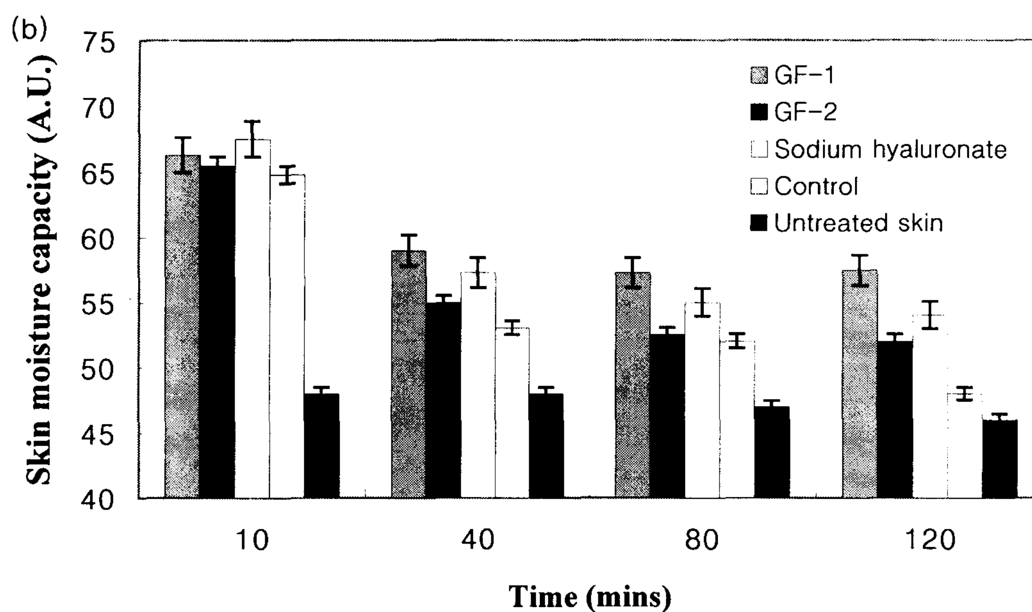
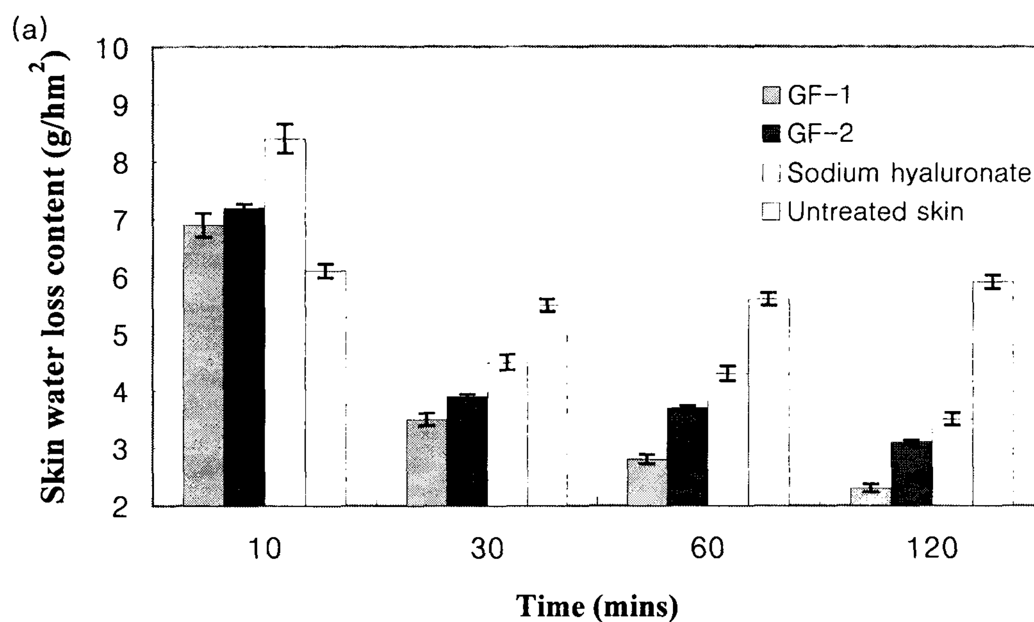


Figure 6. Comparison of Skin moisture capacities by various polysaccharides.

(a) The left forearm of volunteer was treated with each sample (2.0 mg/cm^2).

(b) Treatment of left forearm with O/W emulsion containing 10% at a concentration 0.1% sample, respectively.

Arbitrary capacitance units (A.U., Corneometer CM 825)



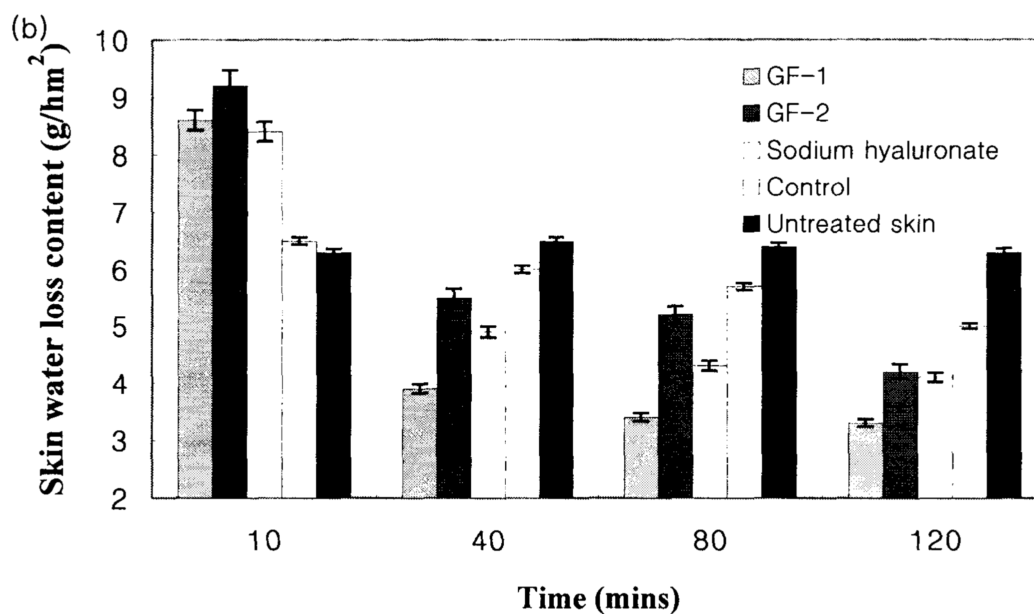


Figure 7. Comparison of transepidermal water loss (TEWL) by various polysaccharides.

(a) The left forearm of volunteer was treated with each sample (2.0 mg/cm^2).

(b) Treatment of left forearm with O/W emulsion containing 10% at a concentration 0.1% sample, respectively.

In conclusion, we confirmed that the GF-1 polysaccharide of fermentation broth was predominant ingredient for cosmetic applications compared to GF-2 polysaccharide of mycelium extract by submerged culture of *G. frondosa.*
These results suggest the GF-1 polysaccharide may be used as ingredients for new moisturizing, anti-aging cosmetic and other biological applications.

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