Master's Thesis of Science in Agriculture

Antimelanogenesis Effects of the Fungal Exopolysaccharides Prepared from the Submerged Culture of *Fomitopsis castanea* Mycelia

검은갓잔나비 버섯 균사체 배양액에서 분리한 세포외 다당체의 멜라닌 합성 조절 기능 연구

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Abstract

Fungal exopolysaccharides are important natural products that exhibit various biological functions, which include anticancer, anti-inflammatory, hypoglycemic, and immunological activities. In this study, exopolysaccharides from *Fomitopsis castanea* mycelia (FEPS) was prepared, and the highest mushroom tyrosinase inhibitory activity was found. FEPS was prepared from cultivation broth by ethanol precipitation method. The extraction yield and protein concentration of FEPS were 213.1 mg/L and 0.03%, respectively. FEPS was found to inhibit mushroom tyrosinase with an IC_{50} of 16.5 mg/mL and dose-dependently inhibited cellular tyrosinase activity (63.9% at 50 μg/mL, and 83.3% at 100 μg/mL) in the cell free extract of SK-MEL-5 human melanoma cell and α-melanocyte stimulating hormone (α-MSH)-stimulated melanin formation in intact SK-MEL-5 human melanoma cell. The IC_{50} of FEPS against NO production from RAW264.7 macrophage cells was 42.8 ± 0.64 μg/mL. By *in vivo* study using zebrafish model, exposure of FEPS at 400 μg/mL to dechorionated zebrafish embryos for 18 h decreased the pigment density, compared to that without FEPS-treated control. Therefore, EPS from *F. castanea* has the potential for cosmetic application as an active ingredient of natural and functional tyrosinase inhibitor.

Keywords: *Fomitopsis castanea*, Exopolysaccharides, SK-MEL-5 human melanoma cells, Zebrafish, Tyrosinase.

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EPS: exopolysaccharides; UV: ultraviolet; TRP1: tyrosinase related protein 1; TRP2: tyrosinase related protein 2; FEPS: isolated EPS from submerged culture of *F. castaneus Imaz* mycelia; NIBR: National Institute of Biological Resources; ZCDM: Zebrafish Center for Disease Modeling; PTU: 1-phenyl-2-thiourea; IDV: integrated density values; L-DOPA: 3,4-dihydroxy-L-phenylalanine; *F. castaneus*: *Fomitopsis castaneus*; PDA: potato dextrose agar; TLC: thin layer chromatography; RPMI: Roswell Park Memorial Institute; DMEM: Dulbecco's modified Eagle's medium; FBS: fetal bovine serum; PBS: phosphate buffered saline; α-MSH: alpha-melanocyte stimulating hormone; hpf: hour post fertilization; NICEM: National Instrumentation Center for Environment Management; SEM: standard error of the mean; NO: nitric oxide; cGMP: cyclic guanosine3'-5'-monophosphate; LPS: lipopolysaccharides

Introduction

1. Mushroom

Mushroom is known as functional foods because of their bioactive compounds that are considered as beneficial for human health [1]. The biological and pharmaceutical activities of mushroom are due to cellular and secondary metabolite compounds, which have been extracted from fruiting body and cultured mushroom medium [1]. In particular, medicinal mushrooms have profound health-promoting effects and had been studied on their medical activity and identifying the bioactive molecules [1].

2. Exopolysaccharides (EPS)

Exopolysaccharides (EPS) are secondary metabolite polymers that secreted into culture medium by microorganism during the growth process. EPS are used in foods, pharmaceuticals, biomedical, and cosmetic industries because of their biological and pharmaceutical activities such as tyrosinase inhibitory activity [2], hepatoprotective effect, antioxidant activity, immunomodulation property [3, 4], anti-inflammatory [5], antitumor, antidiabetic, antimicrobial, and/or immune-stimulatory activities [6] and prebiotic effect [7]. EPS are composed with repeating units of monosaccharides joined by glycosidic linkages [2]. The structure of EPS has a variation in different species due to the difference of monosaccharides and glycosyl linkages in their repeating unit. The difference of EPS structure affects their biological function [9, 10]. The fungal EPS are easy isolation, purification, low making cost, and vast

production in short time compared to EPS prepared from fruiting bodies [9, 10].

3. Submerged fermentation

The technique of conversion from substrates into compounds by microorganisms is called the fermentation [3]. Fermentation can be divided into solid state fermentation and submerged fermentation depending on the substrate type [3]. Submerged fermentation is used as a promising alternative method for the industrial level production of mushroom mycelium and their metabolites [3, 4] compared to field-cultivation that requires several months to cultivate and also needs intensive labor [1]. Compared to solid state fermentation, submerged fermentation provides homogeneous system [4]. The directed synthesis of products is performed by continuously mixing the medium and diffusion of nutrients in the controlled environments [4]. The usage of fermenter makes it possible to control the culture environments by on-line sensors [4].

4. Melanogenesis

Melanogenesis is the process for the production of melanin enhanced by melanogenic enzymes such as tyrosinase and related proteins for protection from UV irradiation [11, 12]. However, overproduction of melanin can cause aesthetic problems [13]. Tyrosinase catalyzes the hydroxylation of L-tyrosine and the oxidation of 3,4-dihydroxy-L-phenylalanine (L-DOPA) [14], and then

the polymerization of pigments [15]. Thus, tyrosinase inhibitors can be used as skin-brightening agents in cosmeceuticals [11].

5. *Fomitopsis castanea*

Fomitopsis castanea is one of wood-decay medicinal fungi species [5]. The purification and fermentation characteristics of EPS from *F. castanea* have been reported [5]. That study focused on the determination of EPS structure and *in vitro* fermentation of EPS in a simulated intestinal environment. The main monosaccharaides of EPS from *F. castanea* were arabinose, mannose, rhamnose, galactose, and glucose [16]. Adding EPS to the fermentation system of the simulated intestinal environment increased the production of short-chain fatty acids content in the fecal extract of tested human [5]. However, there is no report of the melanogenesis inhibitory effects and anti-inflammatory activity of EPS from *F. castanea*. In this regard, we isolated EPS from submerged culture of *F. castanea* mycelia (FEPS), and found that FEPS inhibited melanin biosynthesis and tyrosinase activity. Furthermore, using SK-MEL-5 human melanoma cells and zebrafish, the FEPS inhibited melanogenesis and tyrosinase activity both *in vitro* and *in vivo*, respectively. Therefore, FEPS has the potential to be used as a natural, functional tyrosinase inhibitor in cosmetic application.

Materials and Methods

1. Chemicals and materials

The lyophilized culture supernatants from *Abortiporus biennis, Cryptoporus volvatus, Fomitopsis castanea, Phallus luteus, Pholiota alnicola*, and *Pholiota limonella* mycelia were obtained from the Korea National Institute of Biological Resources (NIBR, Incheon, Korea). Mushroom tyrosinase was purchased from Sigma. SK-MEL-5 human melanoma and RAW264.7 mouse macrophage cell line were purchased from the Korean Cell Line Bank (Seoul, Korea). EZ-Cytox reagent was purchased from DOGEN (Seoul, Korea). Zebrafish eggs were obtained from the Zebrafish Center for Disease Modeling (ZCDM, Korea).

2. Screening of exopolysaccharides from different fungal species against mushroom tyrosinase

The crude EPS from 6 fungal species were prepared by adding 400 μL chilled ethanol to 8 mg of cell free supernatant powder for overnight at 4 ℃. Samples were then centrifuged at 3,200× *g* for 30 min at 4 ℃. The protein was removed using the Sevag method [6]. Briefly, chloroform and n-butyl alcohol (4:1, v/v) were added to exopolysaccharides, then the mixture was vigorously mixed at vortices for 30 min, and centrifuged at 5,700× *g* for 20 min. The supernatants were carefully transferred to new Eppendorf tubes. The inhibitory effect of EPS against mushroom tyrosinase was carried out using reaction mixture containing 10 U/mL mushroom tyrosinase (Sigma), 3.3 mM

L-DOPA (Sigma), and 10% of fungal EPS in 50 mM postassium phosphate buffer (pH 6.8) for 10 min at 25 ℃. The absorbance of the reaction was measured at a wavelength of 475 nm using a SpectroMax M3 microplate reader (Molecular Devices, USA).

3. Preparation of exopolysaccharides from *F. castanea* using submerged fermentation

F. castanea was grown on potato dextrose agar (PDA) medium in a Petri dish at 25 ℃ for 7 days. Then, *F. castanea* was transferred to the seed culture broth, by punching out 5 mm of the agar culture using sterilized cork borer. The seed culture was grown in 200 mL DY media containing 2% (w/v) dextrose and 0.2% (w/v) yeast extract in a 500 mL flask at 25 ℃ at 150 rpm for 7 days. 10% (v/v) of the seed culture was transferred into 1 L culture media in a 1.6 L stirred-tank fermenter (Hanil Inc., Gimpo, Korea) at 25 ℃ and 150 rpm for 18 days. The culture broth from the fermenter was filtered through 110 mm diameter filter paper (Whatman No. 5, Camlab UK). The culture filtrate was then precipitated with four volumes of cold absolute ethanol, stirred vigorously, and kept overnight at 4 ℃. The precipitated EPS were centrifuged at 3,369× *g* for 30 min at 4 ℃, and then dissolved in water, and deproteinated by the Sevag method. The deproteinized polysaccharides were lyophilized at -10℃ under 10 Pa (Eyela FD-550, Tokyo, Japan), and stored at −20 ℃ for further study. The extraction yield of FEPS was calculated by weighing lyophilized precipitates centrifuged from cell free culture [18]. The protein concentration in FEPS was determined by using

Bradford assay with bovine serum (BSA) as a standard. Briefly, samples or BSA standards (0.1 – 1.0 mg/mL) were added to 96-well plate containing 200 μL of dye reagent (Bio-Rad 5000002) and mixed with for 2 min. After 5 min at room temperature, the absorbance was read at 595 nm using a SpectroMax M3.

4. Identification of monosaccharides of exopolysaccharide

FEPS (1 mg) was hydrolyzed using 0.5 M hydrochloric acid at 121 ℃ for 30 min in a vial for the quantification and identification of monosaccharides. The monosaccharide composition of FEPS was determined by thin layer chromatography (TLC). One μL of acid hydrolysate was spotted on a silica gel 60 F_{254} TLC (Merck, Darmstadt, Germany) with glucose, galactose, mannose, arabinose, and rhamnose as standards [17]. The TLC plates were developed five times with acetonitrile: water (85:15, v/v) solvent system. The carbohydrates were then visualized by dipping the plates into a solvent mixture of 0.3% (w/v) N-(1-Naphthyl)ethylenediamine dihydrochloride and 5% (v/v) H_2SO_4 in methanol, followed by 7 min of heating at 121 ℃. The amount of monosaccharides in FEPS was analyzed by conversion to integrated density values (IDV) using the AlphaEaseFC 4.0 program (Alpha Inotech, San Leandro, CA, USA).

$$\text{Monosaccharide (\%)} = \frac{IDV\ of\ monosaccharide}{IDV\ of\ total\ monosaccharide\ in\ FEPS} \text{ x } 100$$

5. Mushroom tyrosinase inhibition assay of EPS from *F. castanea*

The inhibitory activity of FEPS against mushroom tyrosinase was carried out as our previous study [7] with kojic acid as a positive control. Assays were completed as above with FEPS concentration from 0–20 mg/mL FEPS. The absorbance of the reaction was measured at a wavelength of 475 nm using a SpectroMax M3. The inhibitory effect of FEPS on mushroom tyrosinase was calculated using the following equation [8]:

$$\text{Mushroom tyrosinase inhibitory activity (\%)} = 100 - [(S-S_0)/(C-C_0)] \times 100$$

where S is the absorbance of the test sample (enzyme, inhibitor, buffer, and substrate) after reaction, S_0 is the absorbance of the test sample at time zero, C is the absorbance of control (enzyme, buffer, substrate) after reaction, and C_0 is the absorbance of control at time zero. The 50% inhibitory concentration (IC_{50}) was defined as the concentration of FEPS necessary to reduce mushroom tryosinase activity by 50%, relative to a reaction mixture without inhibitor.

6. Cell cytotoxicity tests

SK-MEL-5 human melanoma cells and RAW264.7 mouse macrophage cells were maintained in RPMI 1640 and Dulbecco's modified Eagle's medium (DMEM, GenDepot, USA), supplemented with 10% (v/v) fetal bovine serum (FBS, Gendepot, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, USA) at 37 ℃ in 5 % CO_2 [9]. Cells grown in 96-well plate at 2

× 10^4 cells/well were treated with various concentration of FEPS (0–250 μg/mL) in DMEM medium without FBS at 37 ℃ for 24 h. Then, 90 μL of culture medium was mixed with 10 μL of Ez-CyTox solution. After 1 h at 37 ℃, the absorbance was measured at 450 nm using SpectraMax M3. Percent viability was determined as cell viability relative to the control.

7. Cellular tyrosinase activity assay

SK-MEL-5 human melanoma cells grown in a 6-well plate at 2 × 10^5 cells/well were treated with different concentration of FEPS (50–100 μg/mL) and 500 nM alpha-melanocyte stimulating hormone (α-MSH) for 24 h. Then, the cells were harvested after trypsin treatment, and washed twice with ice-cold PBS. Cell pellets were obtained by centrifugation at 12,600× *g* for 10 min and lysed in 50 mM PBS containing 1% Triton X-100 on ice for 30 min at 4 ℃. Lysates were cleared by centrifugation at 12,600× *g* for 20 min at 4 ℃. The protein concentration in the supernatant was determined using Bio-Rad DC™ protein assay, with bovine serum albumin as a standard. The reaction mixture consisted of cell-extracted protein (10 μg), 2.5 mM L-DOPA in 50 mM PBS buffer (pH 6.8) at 37 ℃ for 1 h. The oxidation of L-DOPA was measured at 475 nm using a SpectraMax M3. Activity was measured using the following formula: Tyrosinase activity (%) = (OD_{475} of sample /OD_{475} of control) x 100.

8. Measurement of nitric oxide production

Nitric oxide production was determined as described previously [10].

RAW264.7 cells grown in 96-well plate at 2×10^4 cells/well at 37℃ for 48 h were treated with FEPS at concentration from 1.56 – 200 μg/mL with 1μg LPS/mL for 24 h and 100 μM indomethacin was used as positive control. Then, culture supernatant (80 μL) was mixed with Griess reagent (80 μL) for 20 min. The absorbance was measured at 540 nm using SpectraMax M3. The amount of nitrite in the sample was calculated from a standard curve prepared with a sodium nitrite standard curve of (0–500) μM in cell culture medium.

9. Zebrafish experiments

Zebrafish embryos were allocated in 6-well plate, with 3 embryos/well of triplicate groups, containing 2 mL of embryos medium, which consisted of 5.03 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2 \cdot 2\ H_2O$, 0.33 mM $MgSO_4 \cdot 2\ H_2O$, and 0.00001% (w/v) methylene blue. At 36 h post fertilization (hpf), FEPS of (100 and 400) μg/mL was treated to each well containing dechorinated zebrafish embryos, and phenotype-based evaluations of anti-melanogenic effects were performed. 1-phenyl-2-thiourea (PTU) and kojic acid (Sigma) were used as positive controls. At 54 hpf, embryos were transferred to the glass bottom for observation and photography of the effect on the pigmentation of zebrafish under the Leica M205FA (Leica Microsystems, Wetzlar, Germany) stereoscope in the National Instrumentation Center for Environment Management (NICEM, Seoul National University, Korea). The quantification of melanin pigmentation was conducted by measuring the estimated raw integrated density on zebrafish embryos using Image J software (National Institutes of Health, USA). The experimental protocols in this study were carried out with

zebrafish larvae up to 54 hpf, and therefore are not subject to the regulations of the Institutional Animal Care and Use Committee [11, 12]

10. Statistical analysis

Experiments were conducted triplicate, and the data were shown as mean ± standard error of the mean (SEM). Statistical analysis was done using one-way ANOVA and Turkey *post hoc* multiple comparison tests on GraphPad Prism 8.0.2 (GraphPad Software, San Diego, CA, USA).

Results

1. Inhibitory effect of different fungal exopolysaccharides against mushroom tyrosinase

The crude EPS from 6 fungal species were examined for tyrosinase inhibitory activity. The crude EPS was extracted by ethanol, and was deproteinized by Sevag method. The aqueous fractions were collected. The mushroom tyrosinase inhibitory effects of EPS that was extracted from lyophilized culture supernatants from *Abortiporus biennis, Cryptoporus volvatus, Fomitopsis castanea, Phallus luteus, Pholiota alnicola,* and *Pholiota limonella* mycelia were shown in Fig. 1. Except for the EPS from *Phallus luteus*, the other EPS inhibited mushroom tyrosinase activity of 1 – 45% of original activity (Fig. 1). Among them, EPS from *Fomitopsis castanea* (FEPS) showed the strongest inhibitory effect against mushroom tyrosinase, and FEPS was selected for further biochemical studies.

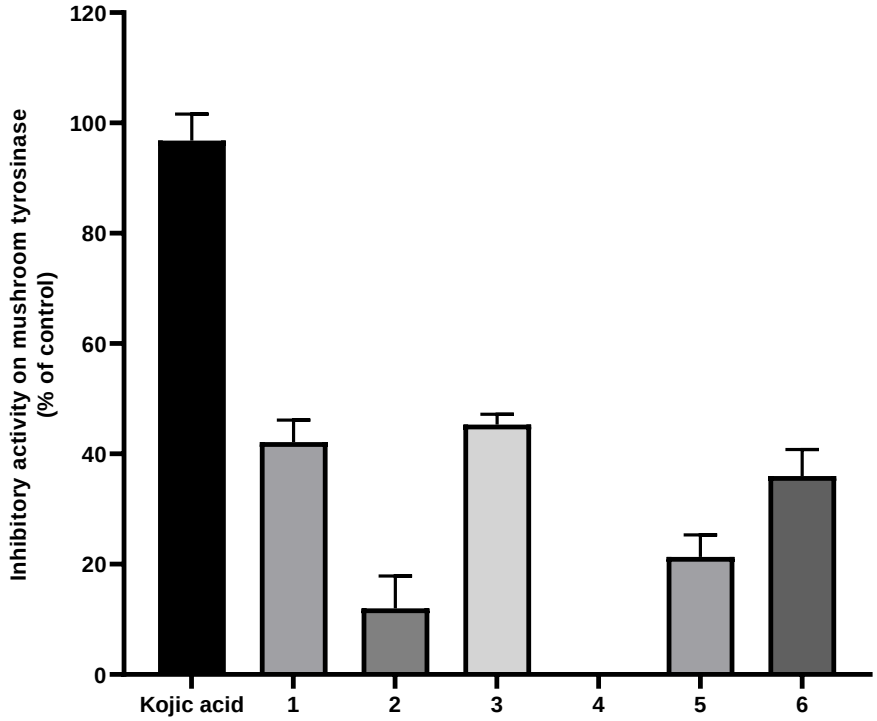


Figure 1. Inhibitory activity of the exopolysaccharides from different fungal EPS against mushroom tyrosinase. EPS extracted from 1: *Abortiporus biennis*, 2: *Cryptoporus volvatus*, 3: *Fomitopsis castanea*, 4: *Phallus luteus*, 5: *Pholiota alnicola*, 6: *Pholiota limonella*. The inhibitory effect of exopolysaccharides against mushroom tyrosinase was carried out at reaction mixture containing 10 U/mL mushroom tyrosinase, 3.3 mM L-DOPA, and 10% of fungus exopolysaccharides in 50 mM postassium phosphate buffer (pH 6.8) for 10 min at 25 ℃.

2. Production of *Fomitopsis castanea* exopolysaccharides using submerged culture

The extraction yield of FEPS was 213.1 mg/L in DY media containing 2% (w/v) dextrose and 0.2% (w/v) of yeast extract. The concentration of protein in FEPS was 0.03%.

3. Monosaccharide composition of *Fomitopsis castanea* exopolysaccharides

After acid hydrolysis, monosaccharide composition was determined by TLC analysis (Fig. 2). Comparing the Rf value of standard compounds obtained from TLC analysis, FEPS was composed of galactose (16.8%), glucose (34.5%), and manose (25.8%) as predominant sugars (Table 1).

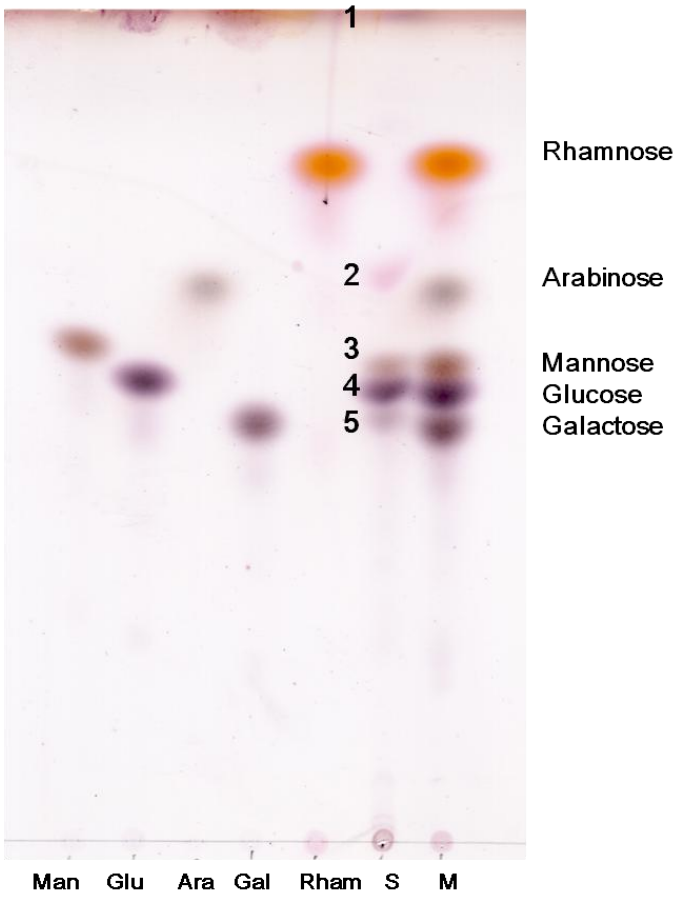


Figure 2. Thin layer chromatogram of monosaccharide composition of FEPS. Man: mannose; Glu: glucose; Ara: arabinose; Gal: galactose; Rham: rhamnose; S: acid hydrolysate of FEPS; M: standard mixtures (mannose, glucose, arabinose, galactose, rhamnose). FEPS (1 mg) were hydrolyzed by 0.5 M hydrochloric acid at 121 ℃ for 30 min in a vial. The monosaccharide composition was analyzed using TLC with five ascents of acetonitrile/water (85:15, v/v).

Table 1. The Rf values of standard compounds, mixed standard, and content of acid hydrolysate of FEPS.

Compound	Standard	Mixed standard	FEPS
Galactose	0.50	0.50	
Glucose	0.55	0.54	
Mannose	0.60	0.58	
Arabinose	0.67	0.67	
Rhamnose	0.82	0.82	
3			0.58
4			0.54
5			0.50

4. Mushroom tyrosinase inhibitory effect

The detail of inhibitory effect against mushroom tyrosinase activity of FEPS of 0–20 mg/mL was studied (Fig. 3). FEPS at 10, 12, 14, 16, and 20 mg/mL inhibited by 18.6%, 27.4%, 32.4%, 43.8%, and 72.4%, respectively. The IC_{50} of FEPS against mushroom tyrosinase was determined as 16.5 mg/mL. FEPS inhibited mushroom tyrosinase in a dose-dependent way.

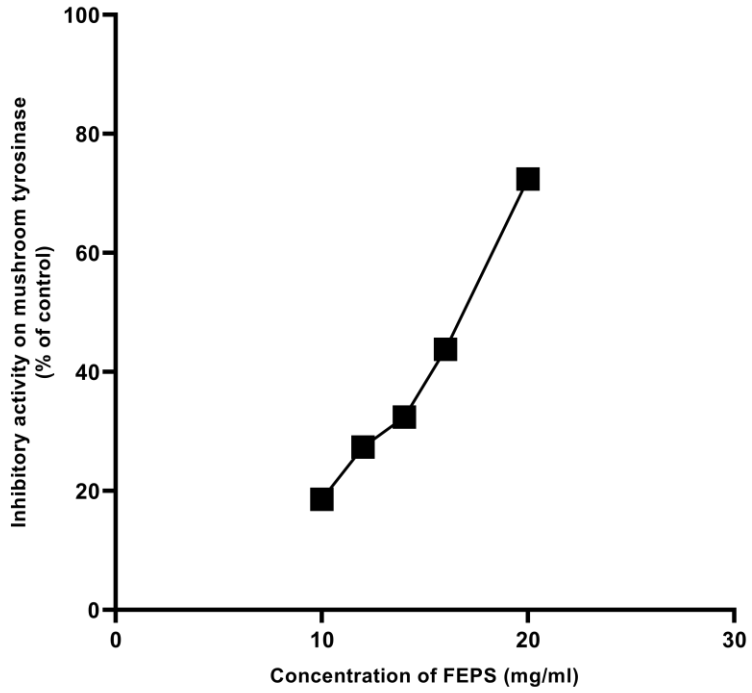
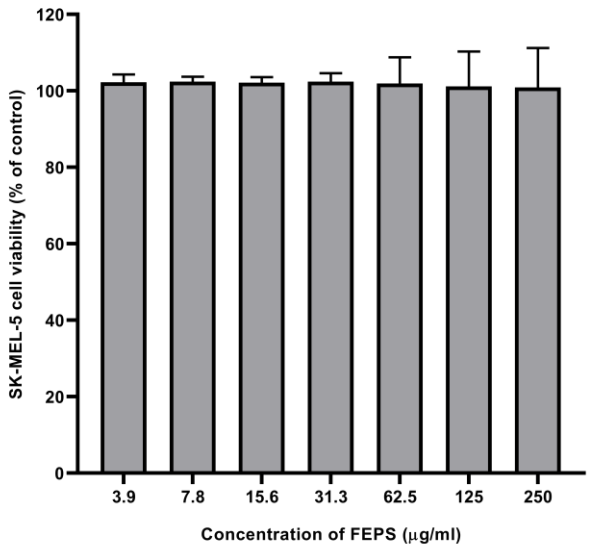


Figure 3. Inhibitory effect of FEPS against mushroom tyrosinase.

5. Cellular tyrosinase activity on SK-MEL-5 human melanoma cells

Cell viabilities of SK-MEL-5 human melanoma cells are shown in Fig. 4A. Since the cells exhibited a survival rate of over 95% at 250 μg/mL (Fig. 4A), the cellular tyrosinase was conducted with concentration ranging (50–150) μg/mL. α-MSH was used to study the inhibitory effects of FEPS on the cellular tyrosinase expression level in SK-MEL-5 cells. The cellular tyrosinase activity of cells treated with α-MSH was assigned as 100%. Incubation for 24 h with FEPS of 50 or 100 μg/mL or kojic acid of 150 μM resulted in tyrosinase inhibition of 63.9%, 83.3%, or 83.3%, respectively (Fig. 4B).

(A)



(B)

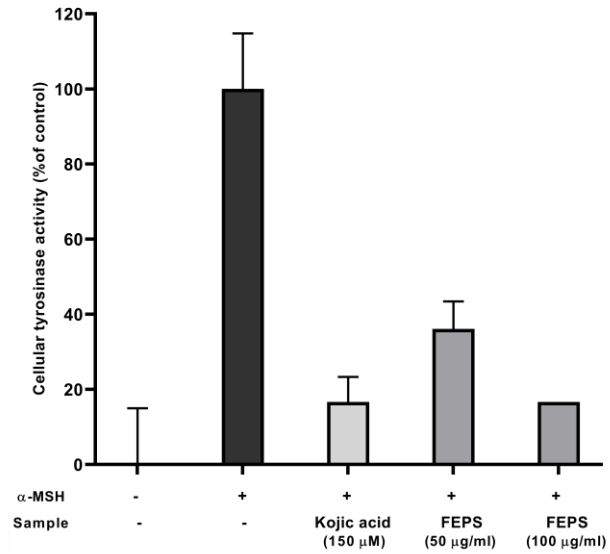
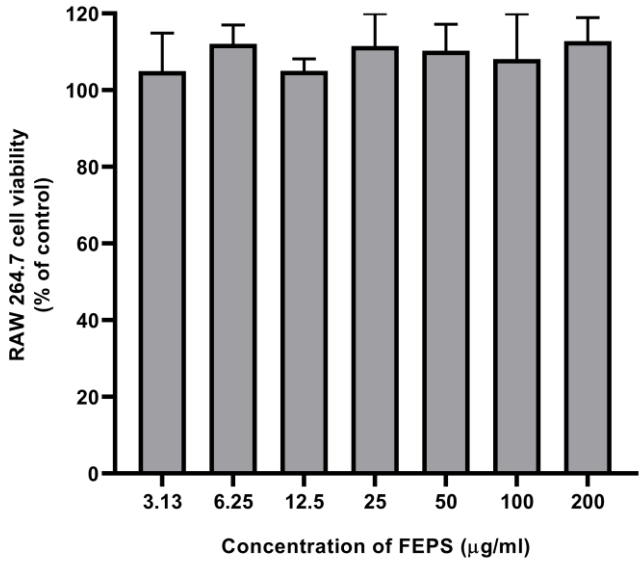


Figure 4. (A) Cell viability, and (B) cellular tyrosinase on SK-MEL-5 human melanoma cells of FEPS**.**

6. Inhibition of nitric oxide production in LPS-stimulated RAW 264.7 cells

In this study, we investigated whether FEPS acted as an inhibitor or scavenger for nitric oxide (NO), and as an inflammatory mediator released from lipopolysaccharide-induced mouse macrophage cell model [13]. Production of NO was investigated after lipopolysaccharides (LPS) stimulation. Since the cells exhibited a survival rate of over 95% at 200 μg/mL (Fig. 5A), the inhibition of NO production in LPS-stimulated RAW264.7 cells was conducted from 3.125 – 200 μg/mL (Fig. 5B). LPS led to an increase in NO production when compared with the negative control, but FEPS at concentration 3.125, 6.25, 12.5, 25, 50, 100, and 200 μg/mL caused a significant reduction in NO production when compared with the negative control. In detail, the inhibitory effect on making of NO in RAW264.7 treated with FEPS at concentration 3.125, 6.25, 12.5, 25, 50, 100, and 200 μg/mL for 24 h were 12.5 ± 0.8%, 13.8 ± 2.1%, 21.3 ± 3.8%, 40.1 ± 1.5%, 53.9 ± 0.2%, 63.6 ± 0.8%, and 71.6 ± 0.9% as compared to the group treated with LPS only, respectively. The IC_{50} of FEPS against NO production from RAW264.7 macrophage cells was 42.8 ± 0.64 μg/mL (Fig. 5B).

(A)



(B)

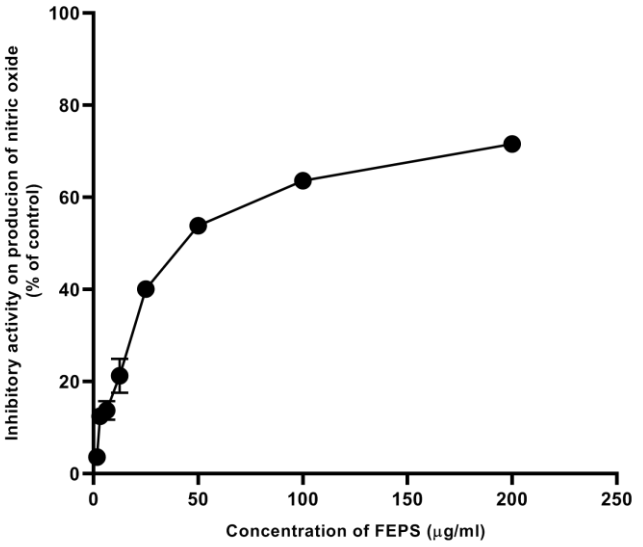


Figure 5. (A) Cell viability, and (B) nitric oxide production on RAW264.7 cells of FEPS**.**

7. Phenotype-based evaluation of melanogenic inhibitory effect using the zebrafish model

Dechorionated zebrafish embryos at 36 hpf were treated with FEPS at 100 and 400 μg/mL, and 10 mM kojic acid and 0.2 mM PTU as positive control (Fig. 6A). The FEPS at 400 μg/mL significantly decreased the pigmentation of dechorionated zebrafish embryos (Fig. 6B). There are no reports on the antimelanogenesis effect of EPS on zebrafish embryos.

A

B

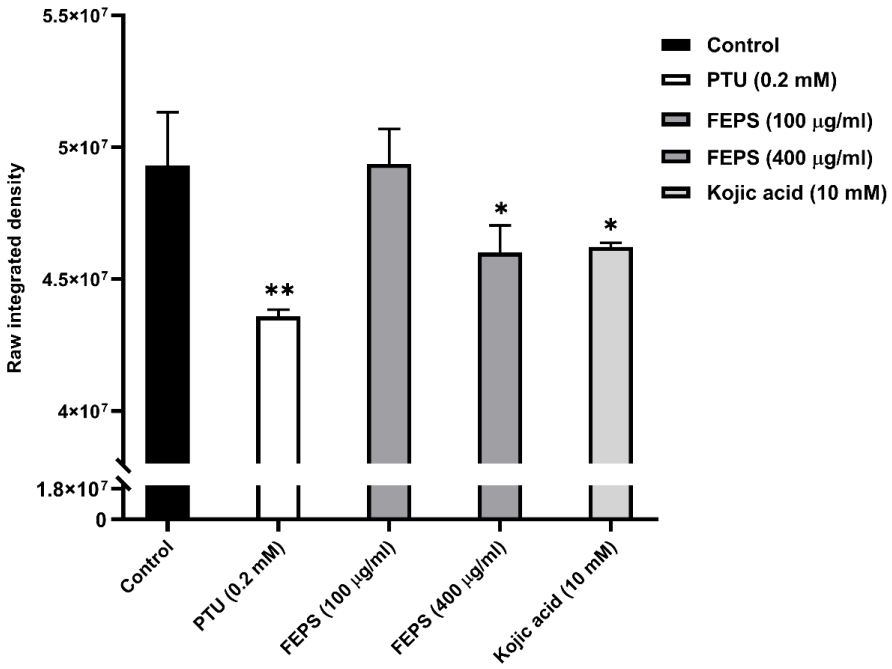


Figure 6. Effect of FEPS on melanin synthesis in zebrafish (A) and the

quantification of melanin pigmentation was conducted using Image J software (B). Dechorionated zebrafish embryos were treated from (36–54) hpf with 10 mM Kojic acid, 0.2 mM PTU, and 100 and 400 μg/mL FEPS, and the effects on pigmentation were assessed using a stereomicroscope. (**a**; control, **b**; 0.2 mM PTU, **c**; 400 μg/mL FEPS, **d**; 100 μg/mL FEPS, **e**; 10 mM kojic acid) (**B)** *$p < 0.1$, **$p < 0.05$ was considered statistical significance compared with the control.

Discussion

Melanin plays an important role in determinant of eye, skin, hair color and human skin homeostasis, such as protecting skin against ultraviolet irradiation, chemical and scavenging toxic drugs [14]. However, abnormal accumulation of melanin in skin as hyperpigmented spots affects the appearance on beauty. To treat skin and pigment abnormalities, many brightening agents have been reported and applied in cosmeceutical, food, and pharmaceutical industries. The natural inhibitors against tyrosinase have increased because safety is strictly monitored in the food and cosmetic industry. In this study, we examined antimelanogenesis of 6 different fungal exopolysaccharides. Among them, EPS from *Fomitopsis castanea* was selected due to its high tyrosinase inhibitory activity. According to our best knowledge, this is the first report demonstrating anti-melanogenesis of exopolysaccharides from *Fomitopsis castanea*. The type of strain, medium components, and physical conditions maintained during fermentation have effects on amount of polysaccharide production, composition of final products, structure, molecular weight, and functional properties of EPS [15-17]. The protein concentration in FEPS is lower than the protein concentration in FEPS extracted by Guo and Chi [5]. The predominant sugars of FEPS in this study were galactose (16.8%), glucose (34.5%), and manose (25.8%), while Guo and Chi reported that EPS from *Fomitopsis castanea Imaz* mainly consists of rhamnose (43.4%), arabinose (0.09%), manose (0.13%), glucose (51.32%), and gallactose (5.06%) [5]. There are various factors that affected monosaccharide content in exopolysaccharides such as monosaccharide composition [18, 19], metal ions [20], aeration rate, agitation speed, pH, temperature [21, 22]. Even same strain, the chemical composition and quality of EPS are also influenced by

conditions such as nutrients status and growth phase [23]. In some cases, one strain can produce different exopolysaccharides with different molecular weight; *Bacillus thermoantarcticus* synthesized two different EPSs, one of EPSs was mainly composed of mannose and glucose in a ratio of 1:0.7, and the other was a mannan [24]. In this study, we used DY media containing 2% (w/v) dextrose and 0.2% (w/v) of yeast extracted, while Guo and Chi used 1% (w/v) peptone, 1% (w/v) beef extract, 5% (w/v) yeast extract, 2% (w/v) glucose, 0.1% (v/v) Tween-80, 0.5% (w/v) sodium acetate, 0.2% (w/v) diammonium citrate, 0.2% (w/v) dipotassium phosphate, 0.058% (w/v) of magnesium sulfate, and 0.025% (w/v) of manganese sulfate [5]. In addition, we used crude exopolysaccharides to analyze monosaccharide content instead of purified EPS as reported by Guo and Chi [5]. Therefore, the different monosaccharide content of EPS from *Fomitopsis castanea* between our finding and the previous repost [5] was possibly resulted by various culture conditions.

Mushroom tyrosinase and α-MSH-stimulated SK-MEL-5 human melanoma cells are used extensively for anti-melanogenesis studies of FEPS. In melanocytes, FEPS inhibited cellular tyrosinase in a dose-dependent manner (Fig. 3B). Keratinocytes and other cells surrounding melanocytes release several melanogenic factors, such as nitric oxide (NO) in response to proinflammatory cytokines [25]. In human melanocytes, NO donors have been reported to increase tyrosinase activity and melanin synthesis [25]. The cyclic guanosine 3’-5’-monophosphate (cGMP) pathway is reported to a major mechanism for NO-induced melanogenesis for the up-regulation of tyrosinase gene expression [26]. NO was demonstrated as a paracrine mediator of UV-induced melanogenesis [25]. Although NO has been reported

to play a beneficial role in the physiological process, such as the regulation of neuronal communication [27], they can also react with oxygen, produce reactive molecules, and aggressively attack biomolecules, resulting in inflammation [27, 28]. We found that FEPS inhibit the making of NO in LPS-stimulated RAW264.7 macrophages cells. However, further studies are needed to evaluate the activation of intracellular signaling pathways of FEPS. We also examined the antimelanogenesis effect of FEPS on zebrafish embryos. The zebrafish embryo is a general vertebrate model system for biochemical researches with high physiological and genetic correspondence to mammals such as melanocytes and melanosmes; In addition, the small size, many offspring in each generation, easy maintenance and handling, high efficient drug diffusion through skin and gills [29, 30]. Therefore, zebrafish embryo can replace animal subjects in animal experiments [29-31]. Melanin pigments can be detected on the zebrafish surface, allowing simple observation of the pigmentation process [32]. 100 and 400 μg/mL of FEPS was added to each well containing dechorinated zebrafish embryos and 400 μg/mL of FEPS significantly decreased the pigmentation of dechorionated zebrafish embryos. FEPS could affect the reduction of melanogenesis via suppressing the tyrosinase activity in zebrafish.

Until now, the detail mechanism of the biological activity of exopolysaccharide relating to monosaccharide, glycosidic linkages, chain information, and degree of branching has not been well established. However, there are several reports that the ratio of monosaccharide such as mannose, rhamnose, and fucose are responsible for the bioactivity of exopolysaccharide. For example, mannose rich exopolysaccharide from *Tremella mesenterica* stimulated the immune system through receptors located on macrophage and

anti-inflammatory activity [33]. L-fucose enriched exopolysaccharides displayed anticancer and anti-inflammatory activity [34]. Human cells are also able to recognize such carbohydrates through mannose receptors, thus stimulating cytokine production and enhancing antioxidant activity, anti-cancer activity of polysaccharide [35-38]. Mushroom exopolysaccharides are homoglycans or heteroglycans. They are able to combine with other proteins to make polysaccharide-protein complex. In addition, the phosphate, sulfate and the amount of protein present decided antioxidant activity of exopolysaccharide [39]. Further study will be needed to elucidate the mechanism.

Conclusions

We screened six fungal exopolysaccharides that showed inhibition activity against mushroom tyrosinase. Among them, EPS from *Fomitopsis castanea* was selected, and exopolysaccharides from mycelial submerged culture were prepared with extraction yield of 213.1 mg/L. The protein concentration of FEPS is 0.03%. The major monosaccharides composed were galactose, glucose, and mannose. FEPS inhibited mushroom tyrosinase with an IC_{50} value of 16.5 mg/mL, and inhibited in a dose-dependent manner against cellular tyrosinase expressed in SK-MEL-5. In addition, FEPS inhibited the nitric oxide production of RAW264.7 macrophage cells with IC_{50} value of 42.8 μg/mL. *In vivo* study of zebrafish model showed that FEPS significantly decreased the pigmentation of dechorionated zebrafish embryos. Therefore, EPS from *F. castanea* has the potential for cosmetic application as an active ingredient of natural and functional tyrosinase inhibitor.

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Abstract in Korean

고등균류가 생성하는 세포외 다당체는 항암, 항염증, 혈당조절, 그리고 면역 조절 등의 생리활성 기능을 나타내는 중요한 천연 재료이다. 본 연구에서 검은갓잔나비버섯 균사체 배양액으로부터 얻은 세포외 다당체(FEPS)에서 가장 높은 타이로시나아제에 대한 억제활성을 확인하였다. 균사체 배양액으로부터 에탄올 침전법을 이용하여 얻은 FEPS의 생산량과 단백질 농도는 213.1 mg/L과 0.03% 이었다. 타이로시나아제에 대한 FEPS의 IC_{50}는 16.5 mg/mL 인 것을 확인하였다. α-MSH 를 이용하여 멜라닌 합성을 유도한 SK-MEL-5 사람의 흑색종 세포에서 타이로시나아제 활성에 대한 저해효과는 FEPS의 농도가 높아짐에 따라 증가하는 양상을 확인하였다 (FEPS 50 μg/mL 에서 63.9 %, 100 μg/mL 에서 83.3%). 또한, LPS로 자극된 RAW264.7 쥐 대식세포로부터의 산화질소의 생성에 대한 FEPS의 IC_{50}는 42.8 ± 0.64 μg/mL 임을 확인할 수 있었다. 지브라피쉬 모델을 이용한 생체 내(*in vivo*) 실험에서는 코리온을 제거한 지브라피쉬에 400 μg/mL FEPS 를 18 시간 동안 노출시켰고, 지브라피쉬의 색소 세기를 측정한 결과 FEPS를 처리하지 않은 대조군에 비해 감소된 것을 확인하였다.

주요어: 검은갓잔나비버섯, 세포외 다당체, 사람 흑색종 세포, 지브라피쉬, 타이로시나아제

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