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Production of Exo-polysaccharide from Submerged Culture of *Grifola frondosa* and Its Antioxidant Activity

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Abstract Exo-polysaccharide isolated from the culture of *Grifola frondosa* was modified by sodium periodate ($NaIO_4$) and sodium chlorite ($NaClO_2$) to delete polysaccharide part and phenolic compound, respectively, and was investigated what effect has each part of exo-polysaccharide against 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidative stress in porcine kidney epithelial cells (LLC-PK1). Oxidative stress on LLC-PK1 cell was measured by cell viability, lipid peroxidation, superoxide dismutase (SOD), and glutathione peroxidase (GSH-px) activity. Exposure of LLC-PK1 cells to 1 mM AAPH for 24 hr resulted in significant decrease in cell viability, SOD, and GSH-px action, and significant increase in lipid peroxidation. The treatment of exo-polysaccharide and $NaIO_4$ modified sample protected LLC-PK1 cells from AAPH-induced cell damage such as cell viability, lipid peroxidation, SOD, and GSH-px activity in a dose dependant manner (10, 100, and 500 μg/mL). However, the treatment of $NaClO_2$ modified sample did not affect for cell viability, lipid peroxidation, SOD, and GSH-px activity. The antioxidant activity of exo-polysaccharide was significantly decreased on AAPH-induced LLC-PK1 cell system when phenolic compound was deleted. The antioxidant activity was significantly correlated with the content of phenolic compound of exo-polysaccharide.

Keywords: *Grifola frondosa*, antioxidant, 2,2'-azobis(2-amidino propane) dihydrochloride (AAPH), porcine kidney epithelial cells (LLC-PK1)

Introduction

Oxidative stress is caused by an imbalance between anti-oxidant system and the production of various oxidants such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH) as it is called reactive oxygen species (ROS). Oxidative damages by ROS lead to various pathological events including coronary heart disease, cancer, and aging (1). In particular, lipid peroxidation in biological membranes has attracted much attention in relation to the deterioration of membrane structure and loss of enzymatic functions (2,3). Clinical research showed the possibility that antioxidants reduce the risk of degenerative diseases by inhibiting free radical induced oxidative damage (1). In addition, several studies have examined both natural and synthetic antioxidants for the inhibition of lipid peroxidation in membrane systems. In case of 2,2-azobis(2-amidino-propane) dihydrochloride (AAPH) which is well employed *in vitro* and *in vivo* assay on the protective effects against oxidation, it rapidly generates free radicals by reacting with oxygen molecules at a constant and measurable rate by its thermal decomposition without biotransformation. This reaction also takes place repeatedly with resultant attacks upon various biological molecules, and it induces physio-chemical alterations and cellular damage (4). Therefore, the function of AAPH, which is causes a diverse array of pathological changes, may be a useful assay system for evaluating biological activities of antioxidants in porcine kidney epithelial cells (LLC-PK1), a renal-tubular epithelial cell line (5).

Grifola frondosa is one of Basidiomycete fungus of Polyporaceae family. Fruit body and liquid-cultured mycelium of this mushroom have been reported to contain useful anti-tumor polysaccharides from various fractions. These polysaccharides have been identified as many types of glucans (6-8). In general, the main components having antioxidant activity from natural products such as medicinal plants and Basidiomycetes are some kinds of phenolic compounds including various flavonoids. In recent research, antioxidant effect of some kinds of polymer, such as fucoidan, β-glucan, and glycoprotein, has reported that those polymers have various antioxidant effect such as lipid peroxidation, ROS radical scavenging, and antioxidant enzyme including superoxide dismutase (SOD) and thiol-containg glutathione (GSH) (9-13).

In this study, we isolated the exo-polysaccharide that have antioxidant effect from culture production of *G. frondosa* and carried out chemical modification with $NaIO_4$ and sodium chlorite $NaClO_2$ and then yielded 2 kinds of product of polysaccharide decomposed and phenolic compound decomposed sample, respectively. To examine which part of exo-polysaccharide contributes to antioxidant effect, we evaluated the protective effect by *in vitro* system on AAPH-induced LLC-PK1.

Materials and Methods

Materials and chemicals 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified medium (DMEM), F12 medium and fetal bovine serum (FBS) were purchased from Invitrgen Co. (Grand

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Island, NY, USA). All other chemicals were used as analytical grade. LLC-PK1 cell line was supplied from American Type Culture Collection (ATCC CRC-1392).

Cell culture LLC-PK1 cell line was maintained at 37°C in a humidified atmosphere of 5% CO_2 in culture plates with a 5% FBS-supplemented DMEM/F-12 medium. After approximately 80% confluence had been reached, the cells were seeded into 24-well plates (4×10^5 cells/well). When the cells is attached (after 3 hr later), 1 mM AAPH was added to each of the wells, pre-incubated for 24 hr, and then incubated with the sample of exo-polysaccharide (10, 100, and 500 μg/mL) for 24 hr (14). The proper concentration of AAPH and the incubation time were determined by the preliminary experiment.

Strain and production of exo-polysaccharide *G. frondosa* HB0071 was maintained on potato dextrose agar (PDA) slants at 4°C and subcultured every 3 months. The seed culture was grown in a 250-mL flask containing 100 mL of potato dextrose broth (pH 5.0) at 25°C on a rotary shaker incubator at 120 rpm for 7 days. The fermentation was carried out under the conditions of temperature 25°C, aeration rate 2 vvm, agitation speed 250 rpm, pH 5.5, and working volume 10 L. The fermentation medium was inoculated with 3%(v/v) of the seed culture and then cultivated at 25°C in a 20-L stirred-tank fermenter (B Braun Korea Co., Ltd., Seoul, Korea).

Treatment of $NaIO_4$ and $NaClO_2$ The fermentation broth was centrifuged at 8,000×g for 20 min, and the resulting supernatant was filtered (Millipore, 0.45-μm) and mixed with 4 times volume of absolute ethanol, stirred vigorously and left overnight at 4°C. The precipitated exo-polysaccharide was collected after centrifugation and designed it as $NaIO_4$ treated sample and $NaClO_2$ treated sample, respectively. For $NaIO_4$ treatment, 50 mg of exo-polysaccharide was incubated in 10 mL of 0.1 M $NaIO_4$ at 25°C for 4 hr. Then 1 mL of 20% ethylene glycol was added (15). For $NaClO_2$ treatment, 100 mg of exo-polysaccharide was dissolved in 10 mL of 4% acetic acid with 500 mg of $NaClO_2$ at 70°C for 40 min and then neutralized 3 N NaOH in the cold bath (16). All treated samples were dialyzed against phosphate buffered saline (PBS). Each yield of $NaIO_4$ and $NaClO_2$ treated sample from exo-polysaccharide was 10 (20%) and 55 mg (55%) by dry weight.

Analysis of total phenolic content The concentration of total phenolics in exo-polysaccharide was estimated by the Folin-Ciocalteu method (17). As a brief, aliquots of 0.1 mL of the extract residue dissolved in dimethylsulfoxide (DMSO, 0.1 mg/mL) was added in a test tube with 0.5 mL of Folin-Ciocalteu reagent and mixed thoroughly. After an interval of 3 min, 0.5 mL of 10% Na_2CO_3 solution was added, and the mixture was allowed to stand for 1 hr at room temperature. The absorbance of the mixture was measured at 760 nm. A standard curve using gallic acid was also prepared. Results were expressed as mg/mL of extract of gallic acid equivalents (GAE).

Cell viability The MTT assay of cell viability was experimented following a modified method (18). Cells were plated in 24-well plates at a number of 4×10^5 cells/each well. After the end of culture, 100 μL of MTT solution (5 mg/mL in PBS) was added to each well containing 1 mL medium. After 4 hr of incubation, the media were removed and formazan crystals were solubilized with 300 μL DMSO. The absorbance of each well was then read at 540 nm using a microplate reader.

Lipid peroxidation Lipid peroxidation was measured as thiobarbituric acid reactive substances (TBARS) production (19). Cells (4×10^5 cells/well) in 24-well plates were first incubated with 1 mM AAPH for 24 hr, and then incubated with exo-polysaccharide, $NaIO_4$ and $NaClO_2$ treated each sample for 24 hr 200 μL of each medium supernatant was mixed with 400 μL of TBARS solution then boiled at 95°C for 30 min. The absorbance was measured at 532 nm and TBARS concentrations were extrapolated from the 1,1,3,3-tetraethoxypropane serial dilution standard curve, TBARS values were then expressed as equivalent nM of malondialdehyde (MDA).

Antioxidant assay under AAPH-induced damage Antioxidant assay is used by the modification of Jimenez's method (20). Briefly, cells (5×10^6 cells/dish) in 10 mm dishes were preincubated for 2 hr, 1 mM of AAPH was added to all of the wells, pre-incubated for 24 hr, and then incubated with or without the indicated concentrations of exo-polysaccharide for 24 hr. The medium was discarded and the cells were washed twice with PBS. One mL of 50 mM potassium phosphate buffer with 1 mM ethylenediamine tetraacetic acid (EDTA) (pH 7.0) was added and cells were scraped. Cell suspensions were sonicated on ice 3 times for 5 sec each time and then sonicated cell were centrifuged at 10,000×g for 20 min at 4°C. Cell supernatants were measured at 540 nm for antioxidant effect by glutathione peroxidase (GSH-px) activity (21). One unit of GSH-px was defined as the amount of enzyme that oxidizes 1 nM of NADPH/min. SOD activity was determined by monitoring the method of autoxidation of pyrogallal (22).

Statistical analysis Data were presented as mean± standard deviation (SD). All data were analyzed using analysis of variance (ANOVA) followed by Tukey-Kramer test for comparison. Statistical significance was defined as $p<0.05$.

Results and Discussion

Cytotoxicity Our results showed that all tested samples were not significantly cytotoxic to LLC-PK1 cells, although the sample modified by $NaIO_4$ exhibited more or less cytotoxicity at the concentration of 100 and 500 μg/mL as 82 and 80%, respectively (Table 1). This cytotoxicity is maybe ascribed to the characteristic of phenolic compound at more or less high concentration. Therefore, the cytotoxicity of all the tested samples themselves was not observed.

Protective effect on AAPH-induced LLC-PK1 cell As shown in Fig. 1, cell survival rate was significantly decreased to 14% in cells treated with AAPH alone. The

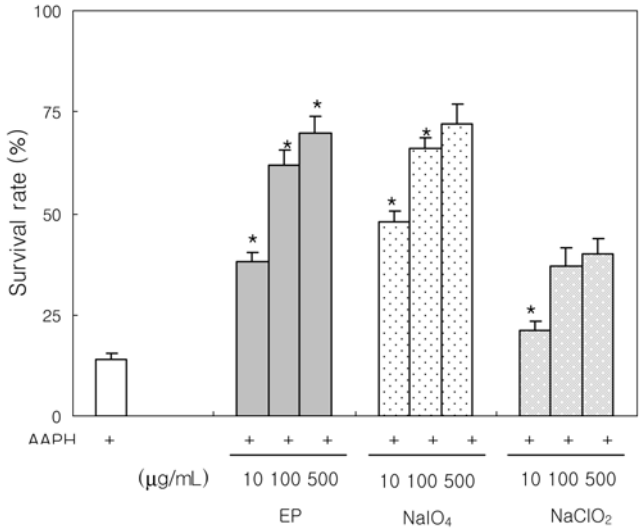

Fig. 1. Protective effect of different concentrations of exo-polysaccharide (EP), $NaIO_4$, and $NaClO_2$ modified samples on AAPH-induced LLC-PK1 cell line. Mean±SEM (n=5), *p<0.05, significantly different compared with 1 mM AAPH.

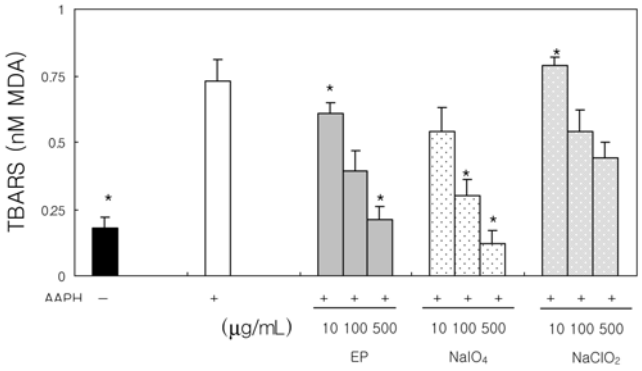

Fig. 2. Inhibitory effect of exo-polysaccharide (EP), $NaIO_4$, and $NaClO_2$ modified samples on lipid peroxidation in AAPH-induced LLC-PK1 cell line. Mean±SD (n=5); *p<0.05, significantly different compared with 1 mM AAPH.

treatment of all the samples from exo-polysaccharide resulted in increase of cell survival rate from 14% to more than 21% at the concentration of 10, 100, and 500 μg/mL. In exo-polysaccharide extracted from the culture of *G frondosa* HB0071 was significantly exhibited protective effect from the damage by AAPH-treatment at the concentration of 10, 100, and 500 μg/mL as 38, 62, and 70%, respectively. In addition, chemically modified $NaIO_4$ sample was exhibited more improved activity by 48, 66, and 72%, than exo-polysaccharide without chemical modification at the same doses, even if high doses of 100 and 500 μg/mL was hardly increased the survival rate due to the cytotoxicity of chemically modified $NaIO_4$ sample in itself as shown as Table 1. However, the treatment of $NaClO_2$ modified sample showed the survival rate of 21, 37, and 40% at the concentration of 10, 100, and 500 μg/mL, respectively. This activity of $NaClO_2$ modified sample was lower activity than exo-polysaccharide and modified $NaIO_4$ sample, even though it also showed meaningful activity on the protection of the damage by AAPH-treatment. This result shows that antioxidant activity of exo-polysaccharide is caused by phenolic compound in this assay, because the decomposition of phenolic compound by $NaClO_2$ was exhibited less successful in protective effect on AAPH-damaged cell line than raw exo-polysaccharide and polysaccharide decomposited sample ($NaIO_4$ modified sample).

Lipid peroxidation To investigate the protective action of exo-polysaccharide, $NaIO_4$, and $NaClO_2$ modified sample against AAPH-induced cytotoxicity, we examined the effects of exo-polysaccharide, $NaIO_4$ and $NaClO_2$ modified sample on inhibition of lipid peroxidation against AAPH-pretreated LLC-PK1 cells (Fig. 2). The lipid peroxidation was markedly increased as almost 4 times in AAPH-pretreated cells, as shown by 0.73 nM MDA in the cells exposed to 1 mM AAPH alone compared with 0.18 nM MDA in untreated cells. However, the treatment of exo-polysaccharide and $NaIO_4$ modified sample significantly dose-dependently and significantly inhibited the lipid peroxidation at the concentration of 10, 100, and 500 μg/mL. Exo-polysaccharide showed significant decrease to 0.61, 0.39, and 0.21 nM MDA in TBARS level at the concentration of 10, 100, and 500 μg/mL. $NaIO_4$ modified sample showed more significant decrease to 0.54, 0.30, and 0.12 nM MDA than exo-polysaccharide in TBARS level at the same doses. Inhibition of lipid peroxidation by treatment of $NaIO_4$ modified sample was more significant than treatment of exo-polysaccharide at all the treated doses. However, the treatment of $NaClO_2$ modified sample showed less successful activity to reducing the level of TBARS at the concentration of 10, 100, and 500 μg/mL as shown by 0.79, 0.45, and 0.44 nM MDA, respectively.

Therefore, the deletion of phenolic compound from exo-polysaccharide showed significant decrease on the indication value of lipid peroxidation compared with both samples which one is the polysaccharide-decomposed and the other is raw exo-polysaccharide without any chemical modification.

Table 1. Cytotoxicity of exo-polysaccharide, $NaIO_4$, and $NaClO_2$ modified samples on LLC-PK1 cell line

Sample	% Cell viability at the concentration (μg/mL)			IC_{50} (μg/mL)
	10	100	500	
Exo-polysaccharide	100±0.5*[1)]	97±0.4*	92±1.5	>500
$NaIO_4$	100±0.2*	82±0.5*	80±0.9*	>500
$NaClO_2$	100±0.9*	97±0.8*	94±2.1	>500

[1)]Values are expressed as mean±SD (n=5); *p<0.05.

Table 2. Effect of exo-polysaccharide, $NaIO_4$, and $NaClO_2$ modified samples on SOD and GSH-px activity in AAPH induced LLC-PK1 cell line

Treatment (μg/mL)		SOD (U/mg protein)	GXH-px (U/mg protein)
Normal		7.42±0.13*[1)]	83.33±5.71*
1 mM AAPH		2.47±0.92*	29.19±2.53*
Exo-polysaccharide	10	3.02±0.09*	34.27±5.01*
	100	4.49±0.11*	59.33±4.41*
	500	6.01±0.53*	63.47±7.37*
$NaIO_4$	10	4.12±0.11*	36.87±4.43*
	100	5.32±0.53*	62.93±5.79*
	500	6.98±0.49*	68.04±7.37*
$NaClO_2$	10	2.79±1.01*	29.98±3.33*
	100	3.17±0.55*	34.57±4.59*
	500	4.07±0.82*	49.17±2.09*

[1)]All values are expressed as mean±SD (n=5); *p<0.05.

Table 3. Total phenolic and flavonoid contents of exo-polysaccharide, $NaIO_4$, and $NaClO_2$ modified samples isolated from *G frondosa* HB0071

Sample	Total polyphenol (mg/g)[1)]	Total flavonoid (mg/g)
Exo-polysaccharide	117±8.12*[2)]	28.4±0.22*
$NaIO_4$	115±10.01*	29.7±0.79*
$NaClO_2$	ND	ND

[1)]All expressed as mg of GAE/g dry weight of residue.
[2)]Each value is expressed as mean±SD (n=3); *p<0.05; ND, not detected.

Antioxidant enzyme activity against AAPH-induced cells Cells are protected from activated oxygen species by endogenous antioxidant enzymes such as SOD and GSH-px. Each effect of exo-polysaccharide, $NaIO_4$ and $NaClO_2$ modified sample on antioxidant enzyme activities in AAPH-pretreated LLC-PK1 cells is shown in Table 2. Pre-treatment with 1 mM AAPH for 24 hr significantly decreased SOD activity of LLC-PK1 cells compared with untreated cells. Each treatment of exo-polysaccharide, $NaIO_4$ and $NaClO_2$ modified sample with LLC-PK1 cells increased SOD activity against 1 mM AAPH-treated cells, even though they didn't show the increase of SOD activity as the same way. In case of the treatment of exo-polysaccharide and $NaIO_4$ modified sample increased the SOD activity at the dose-dependent manner that, in detail, exo-polysaccharide exhibited the SOD activity of 3.02, 4.49, and 6.01 U/mg protein, and $NaIO_4$ modified sample did 4.12, 5.32, and 6.98 U/mg protein at the concentration of 10, 100, and 500 μg/mL, in specific, the treatment of 500 μg/mL of both samples improved SOD activity by around normal level. However, $NaClO_2$ modified sample showed the SOD activity of 2.79, 3.17, and 4.07 U/mg protein, it didn't showed enough activity compared with exo-polysaccharide and $NaIO_4$ modified sample.

GSH-px activity in LLC-PK1 cells treated with 1 mM AAPH was significantly decreased to 29.19 U/mg protein compared with normal level of 83.33 U/mg protein in untreated cells. Treatment of exo-polysaccharide and $NaIO_4$ modified sample resulted in the increase of GSH-px activity on AAPH-pretreated cells at the concentration of 10, 100, and 500 μg/mL, as shown by 34.27, 59.33, and 63.47 U/mg protein in exo-polysaccharide, and 36.87, 62.93, and 68.04 U/mg protein in $NaIO_4$ modified sample. However, GSH-px activity in treatment of $NaClO_2$ modified sample was not significantly increased in AAPH-pretreated LLC-PK cells, as shown by 29.98, 34.57, and 49.17 U/mg protein.

In AAPH-pretreated LLC-PK1 cell system, exo-polysaccharide extracted from the culture of *G. frondosa* HB0071 showed a potential activity on SOD and GSH-Px activities, in specific, the deletion of phenolic compound from exo-polysaccharide interfered to increase activities of SOD and GSH-Px. Therefore, we found that phenolic compounds of exo-polysaccharide significantly contribute to antioxidant activity from these results.

Change of total phenolic and flavonoid contents from exo-polysaccharide Total phenolic and flavonoid contents of exo-polysaccharide were 117 and 28.4 mg/g, respectively, and those of $NaIO_4$ modified sample were 115 and 29.7 mg/g, respectively. However, $NaClO_2$ modified sample for removal of phenolic compounds was not detected any phenoic and flavonoid compound as it is expected. These results showed a significant correlation between antioxidant activity such as lipid peroxidation, SOD and GSH-px and total polyphenol contents in AAPH-pretreated LLC-PK1 cell system. In all assay for cell viability, lipid peroxidation, SOD and GSH-px, the deletion of phenolic compounds from exo-polysaccharide is exhibited lower activity than unprocessed exo-polysaccharide and the deletion of polysaccharide parts. Polyphenols, depending on their precise structure, have been reported to function as hydrogen donating antioxidants, as well as chelators of metal ions, preventing metal-catalyzing formation of initiating radical species (23,24). In this study, polyphenol parts of exo-polysaccharide, which have a potential effect against antioxidant activity, showed significant effect that $NaClO_2$ modified sample, completely deleted phenolic compounds, showed less successful effect on various antioxidant assay using LLC-PK1 cells, even if antioxidant activity was exhibited at high concentration of 500 μg/mL.

Our results also showed a significant decrease of antioxidant enzyme activities such as SOD and GSH-px in LLC-PK1 cells treated with AAPH compared with untreated cells. Exo-polysaccharide of 10, 100, and 500 μg/mL increased significantly the SOD activity. SOD is responsible for the elimination of cytotoxic active oxygen by catalyzing the dismutation of the superoxide radical to oxygen and hydrogen peroxide (25). The superoxide anion radical scavenging activity of SOD is effective only when it is followed by the action of GSH-px, because the activity of SOD generates hydrogen peroxide, which needs to be further scavenged by GSH-px. GSH-px catalyses the reduction of hydrogen peroxide to water, with the simultaneous conversion of reduced glutathione to oxidized glutathione (26).

In conclusion, we demonstrated that exo-polysaccharide from the culture of *G. frondosa* HB0071 can protect LLC-PK1 cells from AAPH-induced cellular damage, which may, in part, be linked to a protective effect of phenolic compounds on lipid peroxidation, SOD and GSH-px activity.

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