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Antitumor and Antioxidant Activities of the Extracts from Fruiting Body of *Phellinus linteus*

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Fruiting bodies of *Phellinus linteus* were extracted by hot water and alkali methods. Sugar contents of PL-H (hot water extract) and PL-A (alkali water extract) were 81.1%, 37.4% and protein contents were 6.2%, 21.8%, respectively. Amino acid pattern showed that two extracts contained large amount of aspartic acid and alanine. Two extracts showed characteristic IR absorption pattern for glycosidic bond at 890 cm^{-1}. PL-H was divided two fractions by gel filtration chromatography and the molecular weights of each fraction were estimated to be about 10 kD and 225 kD, respectively and also PL-A was estimated 10 kD. Two extracts showed strong antitumor, immunomodulating and antioxidant activities, and were compared with commercialized glycopeptide anticancer drugs.

KEYWORDS: Antioxidant, Antitumor, Immunostimulation, *Phellinus linteus*, Polysaccharide

Various mushrooms have been used as a traditional medicine for a long time, the higher Basidomycetes having become a matter of great interest due to their diverse nutritional, medicinal, and pharmaceutical properties. During the past three decades, many polysaccharides and polysaccharide-protein complex have been isolated from the fruiting body and mycelia of mushrooms (Wasser, 2002).

Phellinus linteus, a mushroom growing well on mulberry tree, is well known fungus of the genus *Phellinus* in the family Hymenochaetaceae and has been used as a traditional herb medicine for years in oriental countries (Kim *et al.*, 2004). In the traditional medicine, it has been known to posses curing effect against stomach aches, inflammation, tumors and so on. It is also used to improve overall health and prevent various diseases, such as gastroenteric disorders, lymphatic disease and cancers (Cho *et al.*, 2002). It was first reported in 1968 that the hot water extract from the fruiting body of *Phellinus linteus* inhibited the growth of sarcoma 180 to about 96.7% (Ikekawa *et al.*, 1968), thus a wide variety of further reports have been documented by many investigators (Chung *et al.*, 1993; Chi *et al.*, 1996; Kang *et al.*, 1997; Han *et al.*, 1999). The active polysaccharide from mycelial culture of *P. linteus* stimulates humoral and cell-mediated immunity (Song *et al*,. 1995; Kim *et al.*, 1996). Acidic polysaccharide and proteoglycan from *P. linteus* activate protein tyrosine kinase and protein kinase C (Kim *et al.*, 2003a, c). Ethanol extract showed strong antiangiogenesis and antioxidant activities (Song *et al.*, 2003).

This paper described the evaluation of antitumor, antioxidant activites of polysacchride-protein complex from newly isolated fruiting bodies of *P. linteus* and some information on the polysaccharide-protein complex.

Materials and Methods

Mushroom. The fruiting body of *Phellinus linteus* IY003 was collected at Mountain Bonghwa, Gangwon Province, Korea. The mushroom was authenticated by 5.8S rDNA sequence at Korean Culture Center of Microorganism (KCCM) and a voucher specimen of the mushroom was deposited at the Korean Collection for Type Cultures (KCTC) under the acquisition of KCTC 8927P.

Chemicals. Mesima was purchased from HanKook ShinYak (Nonsan, South Korea), Krestin from Sankyo (Tokyo, Japan), and Cisplatin from Sigma (St. Lousi, MO, USA). All other chemicals and reagents were analytical grade.

Animals and cells. Male ICR mice (20~25 g) and male SD rat (230~250 g) were obtained from Samtako (Osan, South Korea) and kept in stainless steel bottom caged in a room controlled at 23 ± 2°C and 50 ± 5% humidity under a 12 hr dark/light cycle. Sterilized food and water were supplied. Sarcoma 180 and Raw 264.7 cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grandisland, NY, USA) with 10% Fetal Bovine

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Serum (FBS, Gibco, Grandisland, NY, USA) at 37°C and 5% CO_2 in a humidified incubator.

Extraction. The 50 g of fruiting body were cut into small piece and then was extracted 4 times with 500 *ml* water at 121°C for 30 min. After filtration using a 75 μm pore size sieve (ChungGye SangGong, Seoul, South Korea), filtrated solution was concentrated by evaporation at 75°C to 60 *ml*. EtOH (180 *ml*) was added to the concentrated solution, and the suspension was stored at 4°C for 24 hour. After centrifugation (3,000×g), precipitate was dialyzed and lyophilized. The lyophilized extract was designated as PL-H (the extract from the fruiting body of *P. lentius* by hot water method). Other 50 g of fruiting body was extracted by 2 N NaOH 500 *ml*. After neutralization by glacial acetic acid and centrifugation, 3-fold EtOH was added to supernatant, and the suspension was stored at 4°C for 24 hr. After centrifugation, precipitate was dialyzed and lyophilized. The lyophilized extract was designated as PL-A (the extract from the fruiting body of *P. lentius* by alkali method).

Physicochemical assay. The carbohydrate concentration of protein-bound polysaccharide was determined by the phenol-sulfuric acid method (Dubois *et al.*, 1956) using glucose as the standard. Monosaccharide composition in extract was determined by gas chromatography(GC) (Shimazu GL 9A, Kyoto, Japan) equipped with a 3% OV-17 (80~100 mesh) column (2 m × 3 mm) and flame ionization detector. The protein concentration was determined by BCA method (Stoscheck, 1990) using bovine serum albumin as the standard. Composition of amino acid in extracts was determined with Beckman system 6300 amino acid analyzer (Beckman, Fullerton, CA, USA) using acid hydrolysis and ninhydrin procedures. The molecular weight of the extract was determined by gel filtration chromatography on a Sepharose CL-4B column (2.4 cm × 100 cm, Sigma, St. Lousi, MO, USA), using standard dextrans (2000 kD, 500 kD, 124 kD, and 9.3 kD). FT-IR (Bruker IFS-48, Ettlingen, Germany) was employed using the KBr disc for the analysis and detecting functional groups. FT-IR spectrum of curdlan (Sigma, St. Louse, MO, USA) derived from *Alcaligenes faecalis* was used as standard.

Assay of antitumor activity. All aspects of animal care and experiment were performed in accordance with the guide for the care and use of laboratory animals of the National Institute of Health (NIH publication No. 85-23, revised in 1996). Sarcoma-180 tumor cells (5×10^6 cells) were implanted subcutaneously to the mice. The extract treatment started on 3-day after implantation. The extracts (20 mg/kg/day) were administered daily by intraperitoneal injection from 3- to 13-day. The treated mice fed for 30 days, sacrificed, and then tumor extirpated and weighted. The antitumor inhibition percent was calculated using the formula : $(1 - Tw/Cw) \times 100$ (%), where Tw is the average tumor weight (g) of the treated animals and Cw is that of the control animals.

Assay of anticomplementary activity. Anticomplementary activity was measured by the complement fixation test based on complement consumption and the degree of red blood cell lysis by the residual complement. Fifty μl of the extract (50 $\mu g/ml$) was mixed with 150 μl of GVB^{2+} buffer (0.15 mM $CaCl_3$, 0.5 mM, $MgCl_2$, 1.8 mM sodium barbital, 3.1 mM barbituric acid, 141 mM NaCl, 0.1% gelatin, pH 7.4) and 50 μl of guinea pig complement (100 U/*ml*). The mixture incubated at 37°C for 30 min, GVB^{2+} buffer was added to the mixture to final complement concentration (1 U/*ml*). 2 *ml* of antisheep hemolysin (2 MHU/*ml*) sensitized sheep red blood cell (5×10^8 cell/*ml*) was added to the mixture and the suspension was incubated at 37°C for 60 min. The reaction was stopped by adding 70 μl of 0.5 M EDTA and then the stopped reaction mixture was centrifuged at 400×g for 10 min. The 50% of total complement hemolysis (TCH_{50}) was determined at 541 nm. The anticomplementary activity ($ITCH_{50}$) was calculated using the formula : $(1 - Tc/Cc) \times 100$ (%), where Tc is the TCH_{50} of the sample and Cc is that of the control.

Nitric oxide assay. For the determination of nitric oxide (NO) production activity, Raw 264.7 murine macrophage cells (ATCC TIB-71) were seeded in 96 well plates (2×10^6 cell/*ml*), and then incubated with extracts (10 $\mu g/ml$) and positive controls (IFN-γ : 10 U/*ml*, LPS : 10 ng/*ml*) for 24 hr. The amount of stable nitrite, the end products of NO generation by the activated macrophages, was determined by a colorimetric assay. Briefly, culture supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylene diamine dihydrochloride, 2.5% phosphoric acid). This mixture incubated at room temperature for 10 min. The absorbance at 540 nm was read on ELISA reader (Ceres UV, Bio-Tek Instrument, Basel, Switzerland). Nitrite concentration was determined by extrapolation from a sodium nitrate standard curve.

Inhibition of lipid peroxidation. Lipid peroxidation induced by Fe^{2+}-ascorbate system in rat liver homogenate was estimated by thiobarbituric acid (TBA) reaction method. The reaction mixture (2 mg rat liver homogenate, 37.3 mM Tris-cl buffer pH 7.4; 83.5 mM KCl, 10 μM $FeSO_4$; 0.2 mM ascorbate, 1 mg samples, final volume of 1 *ml*) was incubated for 20 min at 37°C. After incubation, the reaction mixture was treated with 0.2 *ml* SDS (8.1%); 1.5 *ml* acetic acid (20%); 1.5 *ml* TBA

(0.8%). The total volume was made up to 5 *ml* by distilled water and kept in a water bath at 95°C for 1 hr. After cooling, 5 *ml* of n-butanol and pyridine mixture (15 : 1, v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4,000×g for 10 min. The organic layer was removed and its absorbance at 532 nm was measured. The result lipid peroxidation was evaluated by the formation of malonaldehyde (MDA). 1,1,3,3-tetramethoxypropane (TMP) was used as standard and butylated hydroxytoluene (BHT) as positive control. The lipid peroxidation inhibition percent was calculated using the formula : (1 − Tl/Cl) × 100 (%), where Tl is the MDA concentration of sample and Cl is that of the control.

Results

Physicochemical properties of extracts. The yield of hot water extracted fraction (PL-H) and alkali extracted fraction (PL-A) were 1.06% and 4.4%, respectively. Sugar contents of PL-H and PL-A were 81.1% and 37.4% and protein contents were 6.5% and 21.8% (data not shown). The contents of sugar and protein were relatively lower, except for the sugar content of PL-H, but this phenomenon is generally observed in polysaccharide-protein complex, the reason is not yet known (Hyun *et al.*, 1990; Ma *et al.*, 1990). Analysis of monosaccharide by GC showed glucose, galactose, xylose, ribose, fructose, mannose as constituent sugars of the polysaccharide. Especially, glucose and galactose were major components of both extracts, but PL-H showed high galactose content than PL-A (Table 1). Sixteen kinds of amino acid were detected and both extracts have aspartic acid and alanine as major components. Most predominat amino acid of PL-H and PL-A were serine and aspartic acid, respectively (Table 2). These amino acid compositions are similar to that of glycopeptide and acidic proteo-heteroglycan from *P. linteus* (Song *et al.*, 1995; Kim *et al.*, 2003b). PL-A appeared as a double peak when subjected Sepharose CL-4B column chromatography and the molecular weight were estimated to be about 10 kD and 225 kD. Both fractions have antitumor properties (data not shown). PL-H has single peak and molecular weight was estimated 10 kD. Both extracts had a low molecular weight compared to other polysaccharide from the fruiting bodies of mushrooms, which generally ranged in size from 10 kD to 1000 kD (Law, 1988).

In order to determine the functional groups of the purified extracts, the FT-IT spectra were measured in KBr pellets. For the sake of comparison, the spectrum of curd-

Table 1. Monosaccharde composition of extracts from *Phellinus linteus* fruiting body

Monosacchardies	PL-H[a] (%)	PL-A[b] (%)
Ribose	7.3	8.2
Xylose	16.1	20.0
Fructose	6.8	7.1
Mannose	6.3	12.7
Galactose	45.6	22.7
α-Glucose	13.9	17.3
β-Glucose	4.0	12.0
Arabinose	Not determined	Not determined

[a]Hot water extract. [b]Alkali extract.

Table 2. Amino acid compositions of extracts from *Phellinus linteus* fruiting body

Amino acid contents	PL-H (%)	PL-A (%)
Asp	11.2	13.5
Tre	10.1	7.4
Ser	19.1	7.1
Glu	6.8	6.9
Pro	6.8	5.6
Gly	10.1	6.8
Ala	13.0	10.0
Val	6.7	7.9
Met	0.6	0.8
Ile	3.9	5.0
Leu	5.1	9.6
Tyr	1.1	2.3
Phe	0.6	3.5
His	5.1	3.7
Lys	3.9	6.6
Arg	2.8	3.1
Cys	Not determine	Not determined

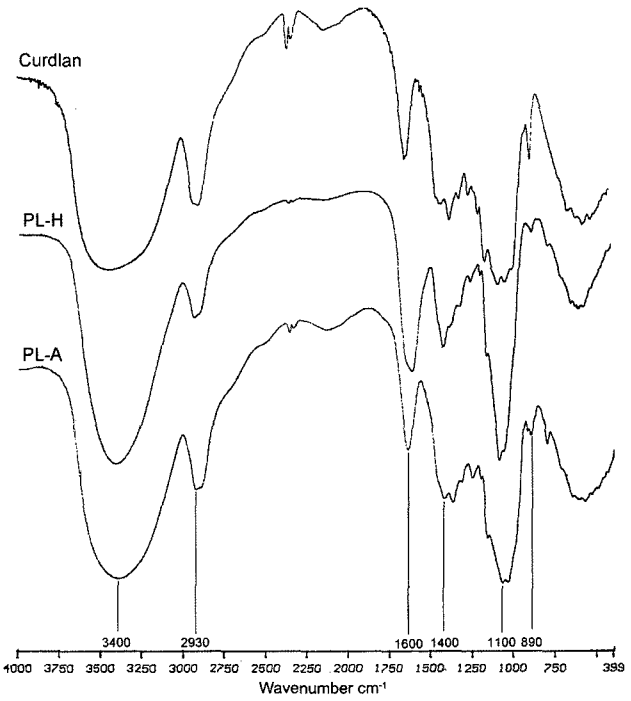

Fig. 1. Comparison of FT-IR spectra of curdlan, PL-H and PL-A.

lan from *A. faecalis* is shown in Fig. 1. The FT-IR spectrum of the two extracts and curdlan, showed the typical characteristics of β-1,3-glucan. The O-H stretching, C-O stretching, C=N stretching, C-H bending and C-O bending were observed at 3400, 2930, 1600, 1400 and 1100 cm^{-1}, respectively. And the characteristic absorption at 890 cm^{-1} in the spectrum were indicative of β-D-glycosidic bond (Zhang *et al.*, 1999; Bae *et al.*, 2002). In comparison with the curdlan, absorption intensity of the 890 cm^{-1} of PL-H and PL-A were small.

Antitumor activity of extracts. Two extract possessed significant antitumor activity against solid tumor model. The 10 consecutive day treatment of PL-H and PL-A (20 mg/kg of body weight) prevented 72.5% and 67.6% of solid tumor growth, respectively. At the same concentration, Mesima, commercialized glycopeptide from *P. linteus*, and Krestin, glycopeptdie from *Coriolus versicolor* prevented 69.6% and 65.7%, respectively. Cisplatin, as commercial most potent anticancer drug, was prevented 30.8% at 2 mg/kg (Table 3).

Anticomplementary activity of extracts. It has been reported that antitumor polysaccharide has anticomplementary activity (Okuda *et al.*, 1972). Moreover, polysaccharide from mushroom is strongly related to the activation of complmentary system (Suzuki *et al.*, 1989). The anticomplementary activity of PL-H showed the highest activity at a concentration of 50 $\mu g/ml$ and attained $ITCH_{50}$ value of 14.5%. At the same concentration, $ITCH_{50}$ value of PL-A, Mesima, Krestin was 4.7%, 6.6%, and 16.4%. Zymosan, well known complementary system inhibitor, was 32.5% (Table 4).

Table 3. Antitumor activity of extracts from *Phellinus linteus* fruiting body

Samples	Dosage (mg/kg)	Inhibition ratio (%)
PL-H	20	72.5
PL-A	20	67.6
Mesima*	20	69.6
Krestin*	20	65.7
Cisplatin*	2	30.8

*; Commercial anticancer drugs.

Table 4. Anticomplementary activity of extracts from *Phellinus linteus* fruiting body

Samples	Concentration ($\mu g/ml$)	Anticomplementary activity ($ITCH_{50}$, %)
PL-H	50	14.5
PL-A	50	4.7
Mesima*	50	6.6
Krestin*	50	16.4
Zymosan*	50	32.5

*; Commercial anticancer drugs.

Nitric oxide (NO) production by extracts. NO is an unique endogenous substance involved in the regulation of a variety of physiological and pathological process (Moncad *et al.*, 1991). NO produced in macrophage by stimulation of IFN-γ and LPS has antiviral, antibacterial, antitumor activity (Hibbs *et al.*, 1988). We have investigated extracts from *P. lentius* on the inducible nitric oxide synthase (iNOS)-mediated NO production in RAW264.7 cells, a murine monocyte/macrophage cell line, with special reference to antitumor activity of against human. In the present study, the basal level of NO in untreated Raw 264.7 was 2.5 μM. After stimulation with the PL-H, PL-A, Mesima, Krestin, IFN-γ and LPS, NO synthesis in the Raw 264.7 increased to the concentration level of 5.7, 14.8, 11.6, 14.2, 34.3 and 41.3 μM, respectively. IFN-γ and LPS were well known NO generating agents (Table 5).

Antioxidant activity of extracts. Antioxidant activities are closely linked with various diseases, aging and food processing and storage. Natural antioxidant may also have correlation with pharmacological actions of plant and microbial source (Song *et al.*, 2003). Antioxidant activities were evaluated for the inhibition of lipid peroxidation (LPO) in rat liver homogenate, initiated by $FeCl_2$. PL-H, PL-A, Mesima, and Kreastin inhibited 55.8, 71.3, 54.0 and 89.0%, respectively. BHT also prevented $FeCl_2$-induced LPO to 92%. Collectively, two extracts definitely possessed strong antioxidant activities (Table 6).

Table 5. Nitric oxide production by Raw 264.7 cell treated with extracts from *Phellinus linteus* fruiting body

Samples	Concentration	Produced NO ($\mu M/ml$)
Control	–	2.5 ± 0.1
PL-H	10 $\mu g/ml$	5.7 ± 0.2
PL-A	10 $\mu g/ml$	14.8 ± 0.6
Mesima	10 $\mu g/ml$	11.6 ± 0.6
Krestin	10 $\mu g/ml$	7.6 ± 0.3
IFN-γ	10 U/*ml*	34.3 ± 1.7
LPS	10 ng/*ml*	41.3 ± 0.9

Table 6. Effect of extracts from *Phellinus linteus* fruiting body on ascorbiate/Fe^{2+} induced lipid peroxidation formation on the liver homogenate

Samples	Concentration (mg/*ml*)	Formed malondialdehyde (nM/*ml*)	Inhibiton ratio (%)
Control	–	30.1 ± 0.4	–
PL-H	1.0	13.3 ± 0.3	55.8
PL-A	1.0	8.6 ± 1.0	71.3
Mesima	1.0	13.9 ± 0.2	54.0
Krestin	1.0	3.1 ± 0.1	89.0
BHT*	1.0	2.4 ± 0.1	92.0

*; Butylated hydroxytoluene.

Discussion

Biological response modifiers that modulated the host biological response against tumors have been developed for application in cancer therapy. A number of polysaccharides and protein bound polysaccharides isolated from mushrooms and are clinically used for the treatment of cancer. Krestin, Mesima, Lentinan from *Lentinus edodes* and Schizophylan from *Schizophyllum commune* are sold in Japan and China as anticancer drugs, and are extensively used in these treatment (Fukushima, 1989). High molecular weight polysaccharide especially glucan are found to stimulate both non-specific host resistance and specific immunological reactivity against tumors (Jong *et al.*, 1991).

In this study, we are using the hot water and alkali extraction method for preparation of antitumor substances. The FT-IR spectrum of the two extracts showed the typical characteristics of b-1,3-glucan. However, the yield of PL-A was higher than that of PL-H. Moreover, contents and compositions of sugar and protein of extracts were distinctive. Molecular weight of PL-A was detected as two peaks by gel filteration, on the other hand, PL-H showed single peak. These observations show that PL-H and PL-A were different polysaccharides from *P. lentius*.

Previous studies by others and ourselves, have shown that polysaccharides isolated from many mushrooms was effective in antitumor and immunostimulation activity. The results of the present investigation demonstrate the antitumor, anticomplementary, NO production and LPO inhibition activities of extracts from *P. lentius*. Results also reveal that PL-H has higher antitumor and anticomplementary activity than those of PL-A, Mesima and Krestin. In comparison, PL-A showed higher NO production and LPO inhibition than those of PL-H, Mesima and Krestin. These results confirmed that PL-H and PL-A might be polysaccharides with different characteristics.

In conclusion, two extracts from *P. lentius* possessed significant antitumor, immunomodulating and antioxidant activities comparable to the activities of commonly used anticancer drugs, such as Mesima and Krestin. However, further studies are needed to elucidate relationship between those activity and pharmacological activity of *P. lentius* and submerged cultivation for industrial potential.

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