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Studies on Constituents of Higher Fungi of Korea (LXVII)
Antitumor Components of the Basidiocarp of *Ganoderma lucidum*

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한국산 고등 균류의 성분 연구(제 67보)
영지버섯 자실체의 항암성분

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ABSTRACT: In order to find physiologically active components from higher fungi, hot-water soluble components were extracted from the basidiocarps of *Ganoderma lucidum*. The extract was purified and separated by DEAE cellulose ion exchange chromatography and Sepharose CL-4B gel filtration method. The separated fractions were designated CR, IN, IA, GL and GH. Fraction GL showed the highest antitumor activity among the fractions and its molecular weight was found to be 47 KD. The tumor inhibition ratio of Fr. GL was 81% at the dose of peritoneal administration of 20 mg/kg/day for 10 days in mice. Chemical analysis of this fraction showed 82% polysaccharide, 8% protein and 0.9% hexosamine. The polysaccharide moiety consisted of 63% glucose, 27% galactose, 7% mannose and 3% fucose. Fraction IN was found to increase the amount of superoxide anion in activated macrophages to 1.6-fold and the number of plaques in hemolytic plaque assay to 6-fold, respectively. These results indicate that the antitumor activity was exerted through immunopotentiation, but not through direct cytotoxicity against the tumor.

KEYWORDS: *Ganoderma lucidum*, antitumor activity, protein-bound polysaccharides, immunopotentiation, superoxide anion, hemolytic plaque assay

Polysaccharides with antitumor activity have been isolated from various natural sources, including higher plants (Nakahara *et al.*, 1964), fungi (Chihara *et al.*, 1970), yeasts (Bradner *et al.*, 1958) and bacteria (Kato *et al.*, 1981). Since Ringler *et al.* (1957) reported the antitumor activity of Basidiomycetes in 1957, studies on the antitumor polysaccharides of Basidiomycetes have been extensively carried out. The antitumor polysaccharides differ widely in their sugar compositions and consequently differ greatly in chemical structures. They can be extracted with various solvents but extracted components can differ in variety with the choice of solvents. One common point of the active polysaccharides is their relatively high molecular weight. These components are considered to exert their antitumor activity through potentiation of the host immunity rather than direct cytotoxicity to tumor cells. It is widely accepted that activated macrophages, cytotoxic T cells, natural killer cells and antibody dependent cytotoxic cells usually play important roles in tumor immunity (Maeda *et al.*, 1970; Juji *et al.*, 1971; Fudenber *et al.*, 1981). More than 700 species of Basidiomycetes have been recorded in Korea but investigations on their antitumor components had been scarce. So our laboratory began to investigate antitumor components from the basidiocarps and cultured mycelia of Korean Basidiomycetes such as *Favolus alveolarius* (Chang *et al.*, 1988), *Coriolus versicolor* (Cho

et al., 1988), *Polyporus giganteus* (Kim *et al.*, 1987), *Volvariella bombycina* (Kim *et al.*, 1985), *Agaricus campestris* (Park *et al.*, 1985), *Pleurotus pulmonarius* (Lee *et al.*, 1985) and *Lyophyllum decastes* (Kim *et al.*, 1984) since 1979. The basidiocarps of *Ganoderma lucidum*, called "Young Ji" or "Bull No Cho", belonging to the family of Polyporaceae, are used as a folk medicine in the Oriental countries such as China, Japan and Korea. *Ganoderma lucidum* has long been a popular folk medicine to cure various human diseases such as hapatitis, gastric ulcer, hypertension, hypercholesterolemia, nephritis, bronchitis, insomnia, neurasthenia, asthma and allergy. Since 1977, a series of studies on *Ganoderma lucidum* has been made and reported by our laboratory: ergosterol was detected in its basidiocarp (Shim *et al.*, 1978); the protein-bound polysaccharide extracted from its basidiocarp showed 87.6% inhibition ratio against sarcoma 180 cells implanted in mice (Kim *et al.*, 1980); its mycelia were artificially grown in submerged culture and were found to produce the antitumor component, its antitumor activity being exerted through immunopotentiation (Kang *et al.*, 1981); seven inorganic elements were identified in its basidiocarp by inductively coupled plasma atomic emission spectrophotometry (Shin *et al.*, 1985); the extract of its basidiocarp showed neither acute nor subacute toxicity in mice (Kim *et al.*, 1986); the morphology of its basidiospore was observed by scanning electron microscopy and the aqueous extract of its basidiocarp prolonged the life span of the mice carrying sarcoma 180 (Lee *et al.*, 1986). The present study was concerned with the separation and purification of the antitumor components of its basidiocarp by ion exchange chromatography and gel filtration, with the chemical characterization of the components and the elucidation of mechanisms of its antitumor activity.

Materials and Methods

Materials

The basidiocarps of *Ganoderma lucidum* (Fr.) Karsten (the family Polyporaceae) used in this study were those cultivated artificially in Kyeong Gi Province in Korea (Fig. 1).

Fig. 1. The basidiocarps of *Ganoderma lucidum*

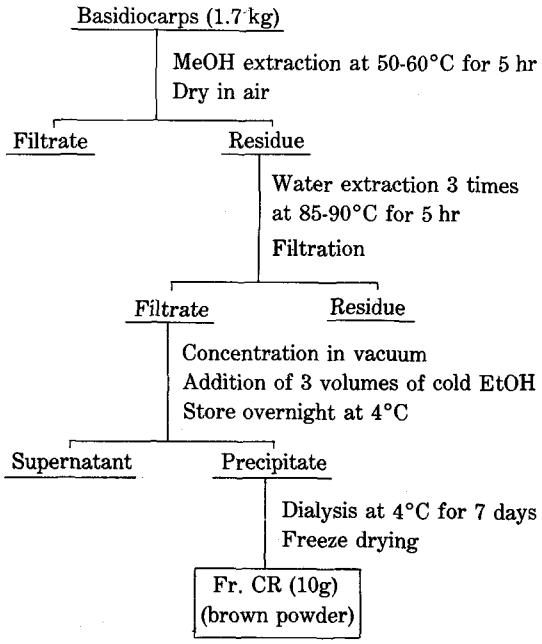
Extraction and separation of basidiocarps

The basidiocarps (1.7 kg) of *Ganoderma lucidum* were disintegrated, immediately washed with 90% methanol and extracted with distilled water at 85-90°C for 5 hr. After filtration, this process was repeated twice. The filtrates were concentrated under vacuum and three volumes of 95% ethanol were added to the concentrate. To complete the precipitation, the mixture was allowed to stand at 4°C overnight. After supernatant was decanted, the precipitate was dissolved in distilled water and dialyzed at 4°C for seven days using visking tube. The precipitate was removed by filtration. The filtrate was concentrated and lyophilized to obtain a brown powder with yield of 10g. It was designated Fraction CR (Scheme 1).

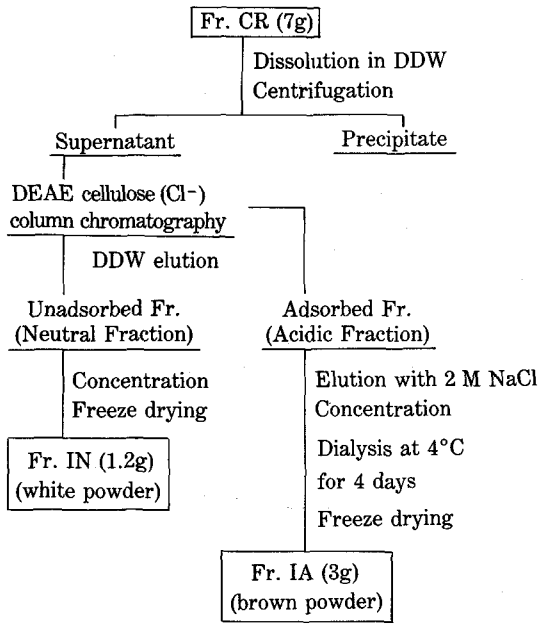
Purification of the water soluble fraction

DEAE cellulose resin (Sigma Chem. Co., USA) was swollen with deionized distilled water (DDW) for overnight. The cellulose resin was washed with two volumes of 0.4 N HCl solution. After decantation, the resin was washed with two volumes of DDW and 0.4 N NaOH solution respectively. This process was repeated three times. After washing, the resin was exchanged to chloride form using 0.4 N HCl and was packed into a column (6×50 cm) with DDW.

A solution of Fr. CR (7g) in 50 m*l* of DDW was applied to the column three times. It was eluted with DDW and 2 M NaCl solution respectively at the flow rate of 90 m*l* per hour. Optical densities of the fractions were measured at 625 nm (Anthrone test) and 660 nm (Lowry-Folin method). Anthrone positive fractions were collected and concentrated. They were dialyzed at 4°C for 3 days and lyophilized. The unadsorbate on the cellulose resin was eluted with DDW and a white powder of 1.2g was obtained. This neutral fraction was designated Fraction IN.



Scheme 1. Extraction and separation of antitumor fractions from *Ganoderma lucidum*



Scheme 2. Isolation of the neutral and acidic fractions from Fr. CR by DEAE cellulose ion exchange column chromatography

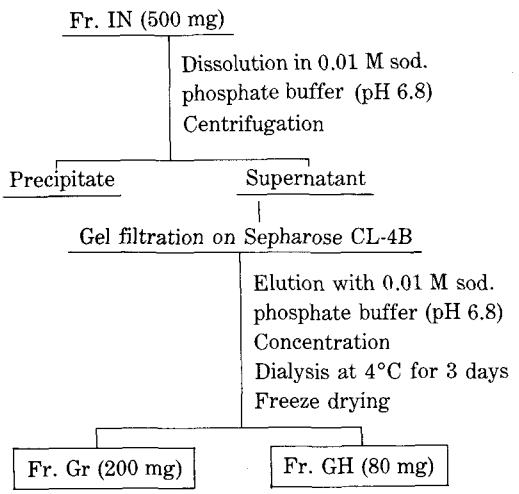
The adsorbate was eluted with 2 M NaCl solution and a brown powder of 3g was obtained. The acidic fraction was designated Fraction IA (Scheme 2).

Molecular weight determination of the antitumor fractions

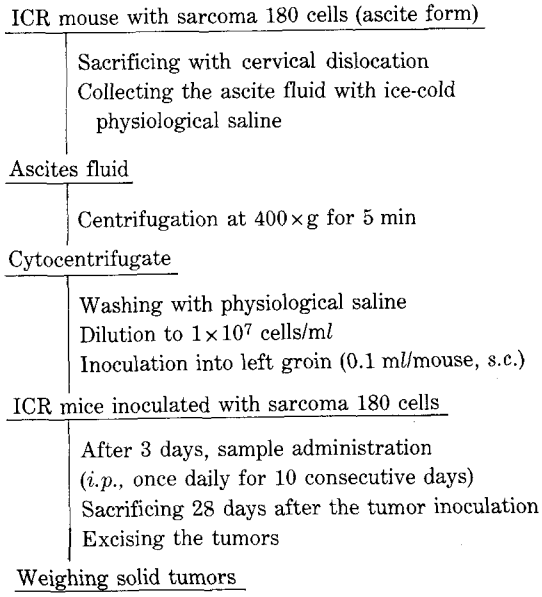
Using Sepharose CL-4B (detection range of molecular weight: 10^3-10^7, Pharmacia Fine Chemicals, Sweden), gel filtration method was adopted to determine molecular weight. Blue dextran (Sigma Chem. Co., USA, MW = 2×10^6), T-480 (Sigma Chem. Co., USA, MW = 4.8×10^5), T-60 (Nakarai Chem. Ltd., Japan, MW = 6×10^4) were used as standard dextrans. Preswollen Sepharose CL-4B was washed with two volumes of 0.01 M sodium phosphate buffer (pH 6.8) twice and degassed under vacuum. The gel was packed into a column (2.5 ×85 cm) with 0.01 M sodium phosphate buffer. The blue dextran (10 mg) was dissolved in the eluent and applied to the column. It was eluted with 0.01 M sodium phosphate buffer (pH 6.8) at the flow rate of 60 m*l* per hour (5 m*l*/fraction). Optical densities of the fractions were measured at 625 nm (Anthrone test). T-480 (MW = 4.8×10^5, 10 mg), T-60 (MW = 6×10^4, 10 mg) and Fr. IN (500 mg) were applied to the same process. Fr. IN was also measured at 660 nm (Lowry-Folin method). Then using elution volumes and logarithm values of molecular weights of standard dextrans, molecular weight of Fr. IN was determined. Anthrone positive fractions were collected and concentrated. They were dialyzed at 4°C for 3 days and lyophilized. Each of the fractions was designated Fraction GL (200 mg) and Fraction GH (80 mg). They were white powders (Scheme 3).

Antitumor test

Male ICR mice (20-25g) were supplied from the Experimental Animal Farm of Seoul National University. Sarcoma 180 cells maintained in the peritoneum of male ICR mice were used for antitumor test. An ICR mouse was sacrificed on the seventh day after inoculation of tumor cells and the ascitic fluid was collected. After the cells washed three times with ice cold saline, the cell suspension was diluted to 1×10^7 cells per 1 m*l*. And 0.1 m*l* of this suspension was subcutane-



Scheme 3. Isolation of the high and low molecular weight components from Fr. IN by Sepharose CL-4B gel filtration



Scheme 4. Procedure of antitumor test *in vivo*

ously inoculated into the left groin of ICR mice.

Each (40 mg) of Fractions CR, IN, IA, GH, GL and Krestin was dissolved in 10 m*l* of saline for a dose of 20 mg/kg/day. They were autoclaved at 121°C, 1.1 kg/cm² for 15 min and stored in a refrigerator. Krestin was used as standard antitumor agent and physiological saline for control. Seven mice were used for each group. Peritoneal administration of 0.2 mg of the fraction in 0.1 m*l* was initiated on the third day after the tumor inoculation and continued for ten consecutive days. On the 28th day after the tumor inoculation, the mice were sacrificed and the solid tumors were excised and weighed (Scheme 4). The tumor inhibition ratio was calculated as follows:

$$\text{Tumor inhibition ratio (\%)} = \frac{C_w - T_w}{C_w} \times 100$$

C_w: average tumor weight of the control group
T_w: average tumor weight of the treated group

Chemical analysis

1) Total polysaccharide contents

Polysaccharide contents of the fractions were quantitatively determined by anthrone test. The optical densities of each fraction and standard solutions were measured at 625 nm. A standard curve was made to calculate the total polysaccharide content of each fraction using glucose, galactose, mannose and a mixture (glucose: galactose: mannose: fucose: xylose = 63:24:9:3:1). The total polysaccharide contents were calculated from the curves.

2) Total protein contents

Protein contents of the fractions were quantitatively determined by Lowry-Folin method.

3) Total hexosamine contents

Free hexosamine and N-acetylhexosamine derived from each fraction were quantitatively determined by Elson-Morgan method (Chaplin, 1987).

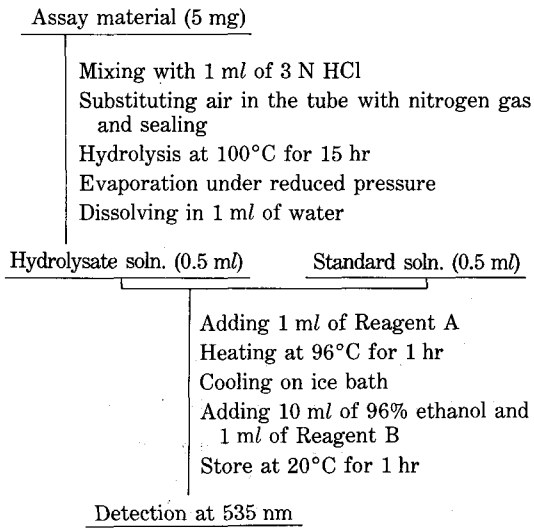
a) Reagents

Reagent A: acetylacetone 1.5 m*l*+ 1.25 N Na_2CO_3 50 m*l*: sample. acetylacetone 1.5 m*l*+ 0.5 N Na_2CO_3 50 m*l*: standard

Reagent B: *p*-dimethylaminobenzaldehyde (1.6g) was dissolved in 30 m*l* of conc. HCl and 30 m*l* of 96% ethanol was added

b) Method

Each fraction (5 mg) was dissolved in 1 m*l* of 3 N HCl and the air in the tube was substituted with nitrogen gas and sealed. Each mixture was hydrolyzed for 15 hr at 100°C and dried. The dried fraction was dissolved in 1 m*l* of H_2O. Each (0.5 m*l*) of Fractions IN, GL and



Scheme 5. Procedure of hexosamine analysis

standard hexosamine solution was added to 1 ml of Reagent A. Each mixture was reacted at 96 °C for 1 hr and cooled rapidly in an ice bath. After cooling, 10 ml of 96% ethanol and 1 ml of Reagent B were added to the mixture. The sample solutions were stored for 1 hr at room temperature and detected at 535 nm. A standard curve was constructed to calculate the total hexosamine contents in each fraction using glucosamine as standard (Scheme 5).

4) Monosaccharide analysis

The sample and standard solution were injected into Shimadzu gas chromatography RIA and analyzed.

5) Amino acid analysis

The sample and standard solutions were injected into Hitachi amino acid analyzer 835 and analyzed.

Instrumental analysis

1) Elemental analysis

Contents of elements in each fraction were quantified by Perkin-Elmer elemental analyzer.

2) IR spectroscopy

Each sample was analyzed by KBr disc method, using Perkin-Elmer IR 20.

Effects of fraction IN on immune responses

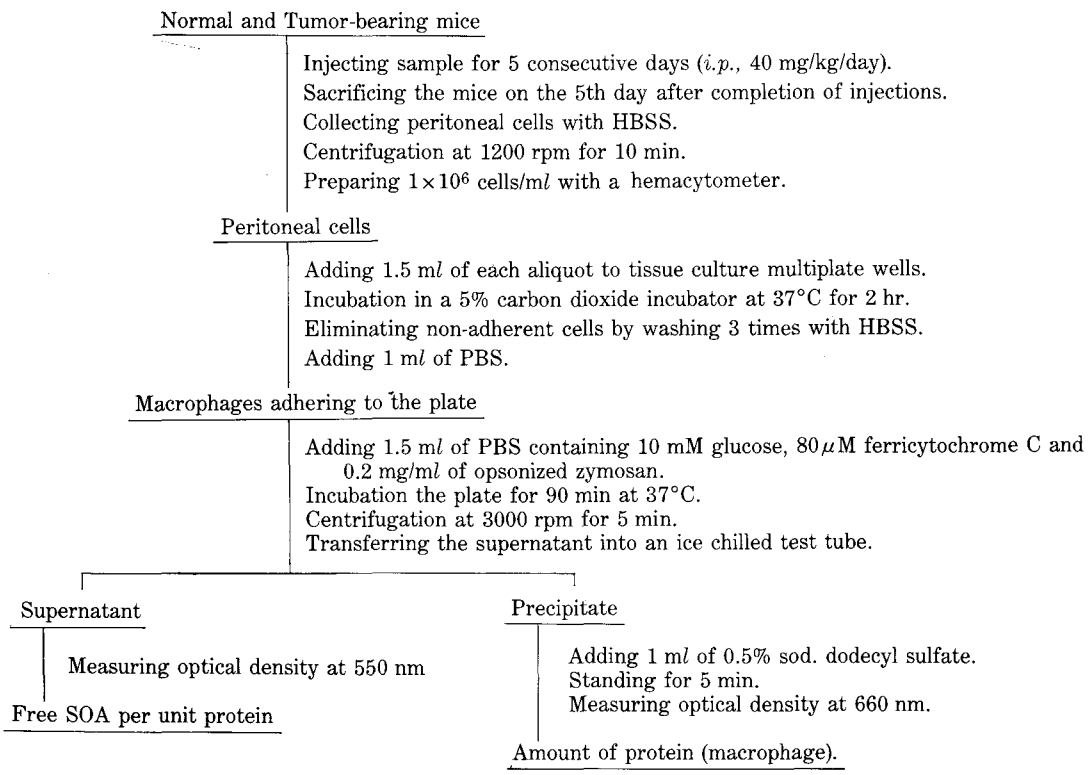
1) Effects on macrophage activation

a) Reagents

Hank's Balanced Salt Solution, pH 7.3 (HBSS), Phosphate Buffered Saline, pH 7.3 (PBS), Zymosan (Sigma Chem. Co., USA), Human serum (Sigma Chem. Co., USA), Ferricytochrome C (Sigma Chem. Co., USA), RPMI-1640 medium (Gibco Co., USA), Tissue culture multiplate well (Falcon Co., USA)

b) Method

Normal and tumor-bearing ICR mice were given 40 mg/kg/day of Fr. IN intraperitoneally for five consecutive days. Peritoneal cells were obtained by washing the peritoneal cavity of mice killed by cervical dislocation with HBSS. After centrifugation at 1200 rpm for 10 min, the precipitated cells were collected and adjusted with RPMI-1640 medium to 1×10^6 cells/ml Aliquots of 1.5 ml each were cultured in an incubator at 37°C for 2 hr in humidified atmosphere of 5% CO_2 in the air so that macrophages were adsorbed selectively on the plate. The non-adherent cells were eliminated by washing 3 times with HBSS. Zymosan was adjusted with PBS to 10 mg/ml, boiled for 1 hr, washed 3 times and resuspended in PBS at 50 mg/ml. A mixture of 1 volume of zymosan (50 mg/ml) and 4 volume of human serum was incubated at 37°C for 30 min. After centrifugation, PBS was added to precipitate zymosan. A 10 mg/ml solution of opsonized zymosan was prepared. On the 5th day after daily injection of the fraction, macrophages were collected and superoxide anion (SOA) was measured by the method described by Ito *et al.* (1983). Then 1.5 ml of PBS containing 10 mM glucose, 80 μM ferricytochrome C and 0.2 mg/ml of opsonized zymosan were added to macrophages adhering to the plate and the plate was incubated for 90 min at 37°C. After incubation, the culture was centrifugated at 3000 rpm for 5 min. The supernatant was transferred into an ice-chilled test tube and the absorbance was measured at 550 nm. On the other hand, 1 ml of 0.5% sodium dodecyl sulfate was added to the precipitated cells and then after standing for 5 min, the cells were well dispersed and the amount of protein was measured by Lowry-Folin method. The amount of ferricytochrome C was obtained from



Scheme 6. Procedure of superoxide anion assay in activated macrophages

the absorbance at 550 nm, according to the formula: $E_{550} = 2.1 \times 10^4\ M^{-1}\ cm^{-1}$ and free SOA per unit protein was calculated (Scheme 6).

$$\text{SOA nM/mg protein} = \frac{\text{OD at 550 nm} \times 15.87}{\text{macrophage amount (mg)}}$$

2) Effects of antitumor components on hemolytic plaque-forming cells

a) Materials and Reagents

DEAE dextran (Pharmacia Fine Chemicals, Sweden), cover glass (4.5×50 mm; Fisher Co., USA), sheep red blood cells (SRBC), complement, tissue culture plate (Green Cross Med. Co., Korea) were used.

b) Methods

Twelve mice were divided into two groups. For a treated group, a dose of 40 mg/kg/day of Fr. IN was injected intraperitoneally once a day for five consecutive days. Physiological saline was used for control group. On the 7th day after the last sample injection, the mice were immunized by intraperitoneal injection of 1×10^7 cells of SRBC. On the 5th day after immunization, the mice were sacrificed with cervical dislocation and the spleens were excised. The spleens were homogenized with ice-cold HBSS and centrifugated at 400×g for 5 min. The cytocentrifugates were collected and hemolyzed with 0.83% NH_4Cl solution at 37°C for 5 min. After hemolysis, the cells were washed three times and centrifugated under the same condition. The cytocentrifugates were resuspended in ice-cold HBSS. The spleen cells were counted directly by a hemacytometer. This procedure (Klaus, 1987) is a modification of the method originally described Jerne *et al.* (1963). Agar (0.5%) was dissolved in HBSS to which is added 0.5% DEAE dextran. The melted agar (0.35 ml) was added in the culture tubes which were held at 47°C. Indicator SRBCs were washed three times with HBSS and the pellet was diluted with equal volume of HBSS. SRBC (25μl) was added to each tube of agar and test splenocytes (150μl) which were diluted to give

ICR mice

Injecting sample for 5 consecutive days (*i.p.*, 40 mg/kg/day).
After 7 days, immunization by injection of SRBC (1×10^7 cells, *i.p.*).
After 5 days, sacrificing with cervical dislocation.
Weighing body weight.
Excising spleen and weighing.

Spleen

Homogenization with ice cold HBSS.
Centrifugation at 400×g for 5 min.
Hemolysis with 0.83% NH_4Cl.
Centrifugation at 400×g for 5 min.

Spleen cells without erythrocytes

Washing 3 times with ice-cold HBSS.
Counting splenocytes with a hemacytometer.

Spleen cells suspension (150 μl)

Adding of 25 μl of 50% SRBC, 25 μl of complement and 350 μl agar soln at 47°C.
Mixing and placing 100 μl of mixture on the culture dish.
Overlaying a microscopic cover glass on the mixture.
Solidifing the agar plate.
Incubation at 37°C for 3 hr.

Counting hemolytic plaque-forming cells

Scheme 7. Assay procedure of hemolytic plaque-forming cells

a proper number of plaques to count were added. The 1:4 diluted guinea pig complement (25 μl) was added and the tubes were immediately vortexed and poured into a petri dish. The agar was overlayed with a cover glass. The agar plates were allowed to solidify and were incubated for 3 hr at 37°C and the numbers of hemolytic plaques were counted (Scheme 7).

$$\text{PFC}/10^6 \text{ spleen cells} = \frac{N}{C\times V_m\times a}\times 10^6$$

$$\text{PFC/total spleen cells} = \frac{\text{PFC}}{10^6 \text{ spleen cells}}\times C\times V_s/10^6$$

$$a = \frac{150 \text{ (Vol. of spleen cell supension)}}{550 \text{ (Vol. of incubation mixture)}}$$

N: number of plaques observed in one cover glass

C: count of spleen cells in 1 m*l* of spleen cell suspension

V_m: volume of incubation mixture filled into one cover glass (m*l*)

V_s: total volume of spleen cell suspension (m*l*)

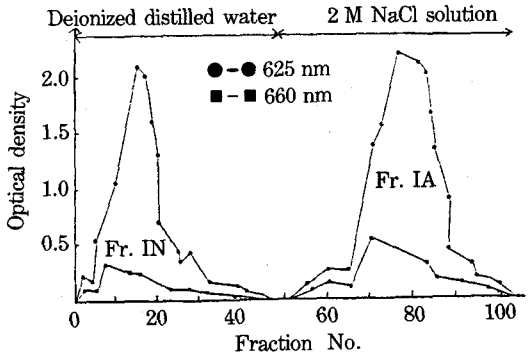


Fig. 2. Elution pattern of Fr. CR by DEAE cellulose ion exchange chromatography

Results and Discussion

Extraction, purification and molecular weight determination of the antitumor fractions

A brown powder (10g), designated Fraction CR, was extracted from the basidiocarps (1.7 kg) of *Ganoderma lucidum*. Fraction CR was applied to DEAE cellulose ion exchange column. The unadsorbed fraction eluted with the deionized distilled water was designated Fraction IN (1.2g) that was a white powder of neutral polysaccharides. The adsorbed fraction eluted with 2 M NaCl solution was designated Fraction IA (3g) that was a brown powder of acidic polysaccharides. The elution profile of Fraction CR was shown in Fig. 2. Molecular weights of the fractions were determined by Sepharose CL-4B gel filtration using blue dextran, T-480 and T-60 as standards. The elution pattern of standard dextrans was shown in Fig. 3. When Fraction IN was applied to Sepharose CL-4B column, it was separated into Fractions GL and GH (Scheme 3). The elution patterns of Fractions GL and GH were shown in Fig. 4. The molecular weight of Fraction GL was 47 KD and that of Fraction GH was 468 KD (Fig. 5).

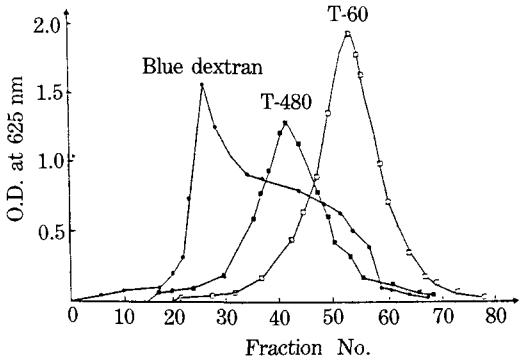


Fig. 3. Elution diagram of standard dextrans by Sepharose CL-4B gel filtration
●-● Blue dextran: dextran (MW 2,000,000)
■-■ T-480: dextran (MW 480,000)
□-□ T-60: dextran (MW 60,000)

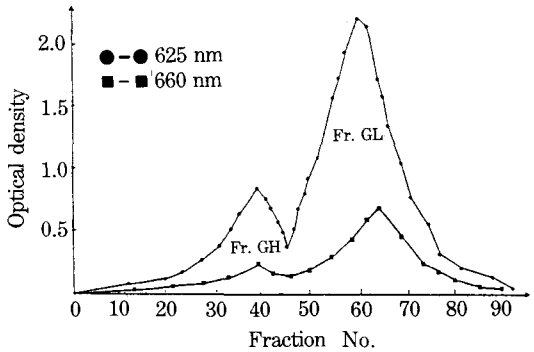


Fig. 4. Elution diagram of Fr. IN by Sepharose CL-4B gel filtration

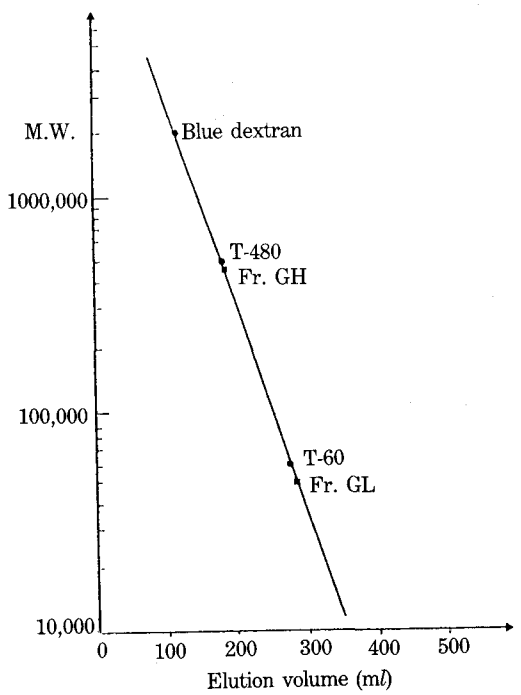


Fig. 5. Determination of molecular weight of Fr. GL and Fr. GH by Sepharose CL-4B gel filtration with standard dextrans

Antitumor activity

Tumor inhibition ratio of each fraction and the comparison with Krestin against sarcoma 180 cell growth in mice were shown in Table I.

Table I. Antitumor activities of the various fractions obtained from *Ganoderma lucidum*

Fraction	Dose (mg/kg/day)	Tumor weight (g) (Mean±S.D.)	Inhibition ratio (%)	Complete regression (No. of mice)
Control	20	1) 6.90±1.5	-	0/7
		2) 6.94±2.7	-	0/7
Krestin	20	1) 2.65±1.2#	62 %	0/7
		2) 2.89±1.4*	59 %	0/7
Fr. CR	20	1) 3.10±1.5*	55 %	0/7
Fr. IN	20	1) 2.36±0.8*	66 %	2/7
		2) 2.45±1.1*	64 %	0/7
Fr. IA	20	1) 3.28±2.2*	52 %	0/7
Fr. GH	20	2) 1.64±0.9*	76 %	1/7
Fr. GL	20	2) 1.30±0.5*	81 %	2/7

The results of two separate experiments (1,2) were summarized in this table.
#) $p<0.01$; *) $p<0.05$

Table II. Polysaccharide and protein contents of the antitumor components

Fraction	Polysaccharide		Protein[c] (%)
	Glu[a] (%)	Mix[b] (%)	
CR	60.28±4.52*	64.36±2.38	3.74±0.62
IN	69.36±6.89	74.38±5.10	5.74±1.24
IA	43.40±4.28	43.11±3.49	2.76±0.43
GL	75.13±8.52	82.25±6.25	7.86±1.67
GH	72.14±9.15	78.35±7.68	6.25±1.53

*Mean±standard deviation
[a] Standard glucose
[b] A mixture of glucose, galactose, mannose, fucose and xylose
[c] Standard bovine serum albumin

Table III. Hexosamine contents of the antitumor components

Fraction	Hexosamine (%)
IN	0.48±0.04*
GL	0.90±0.07
GH	0.84±0.11

*Mean±standard deviation

Table IV. Monosaccharide contents of the polysaccharide moiety of the antitumor components by G.L.C. analysis

Monosaccharide	Fr. CR	Fr. IN	Fr. IA	Fr. GL	Fr. GH
Glucose	62.50	63.90	58.20	62.72	68.36
Galactose	20.70	21.46	27.17	26.76	23.87
Mannose	13.90	10.30	8.55	7.29	5.92
Fucose	2.00	2.24	5.10	3.21	**ND
Xylose	0.86	ND	ND	ND	1.84

*Area percentage
**Not detected

Fraction GL showed the most effective inhibition ratio of 81% among the five fractions at the dose of 20 mg/kg/day. And two of the mice showed complete regression of the implanted tumor.

Chemical composition

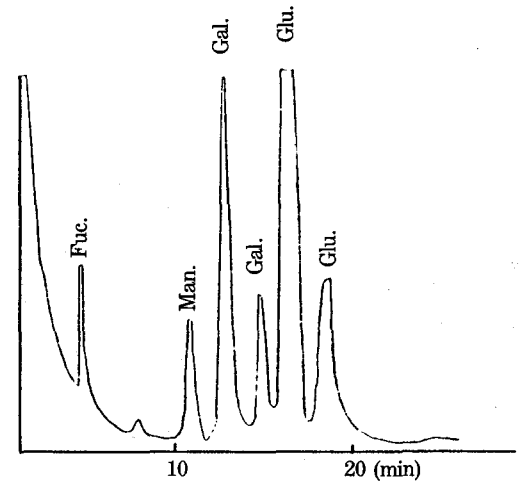


Fig. 6. GLC pattern of the acid hydrolysate of Fr. GL

Table V. Amino acid contents of the protein moiety of the antitumor components

Amino acid	Fr. CR	Fr. IN	Fr. IA	Fr. GL	Fr. GH
L-Aspartic acid	7.52*	3.27	5.59	5.31	5.22
L-Threonine	8.18	9.20	7.30	7.13	4.15
L-Serine	10.32	16.13	10.73	10.53	6.26
L-Glutamic acid	7.54	3.01	6.79	6.35	6.67
L-Glycine	10.41	8.23	9.16	8.98	7.85
L-Alanine	10.88	10.11	7.55	10.13	6.83
L-Cysteine	0.09	0.24	ND**	0.17	ND
L-Valine	8.04	6.64	9.88	6.66	3.83
L-Methionine	0.99	0.58	ND	0.87	0.23
L-Isoleucine	2.98	1.29	1.98	2.36	2.70
L-Leucine	5.21	1.94	2.82	4.08	4.58
L-Tyrosine	1.15	0.28	0.47	0.56	0.35
L-Phenylalanine	2.13	1.27	1.53	2.09	2.49
L-Lysine	4.55	3.08	3.15	4.41	4.41
L-Histidine	2.81	3.42	3.01	2.61	3.16
L-Arginine	2.24	1.00	1.18	2.01	2.27
L-Proline	0.55	0.49	0.53	0.47	0.42

*Area percentage
**Not detected

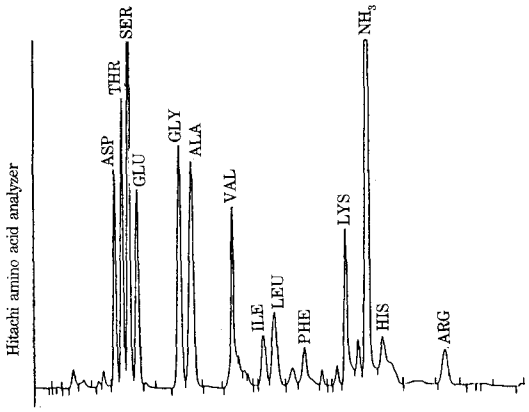


Fig. 7. Chromatogram of amino acids in the hydrolysate of Fr. GL

Table VI. Elemental composition of the antitumor components

Element	C	H	O	N
Fr. CR	6.00*	9.90	5.53	0.82
Fr. IN	6.00	10.32	6.50	0.51
Fr. IA	6.00	10.20	6.83	0.87
Fr. GL	6.00	10.28	6.38	0.64
Fr. GH	6.00	10.38	6.12	0.71

*Mole ratio

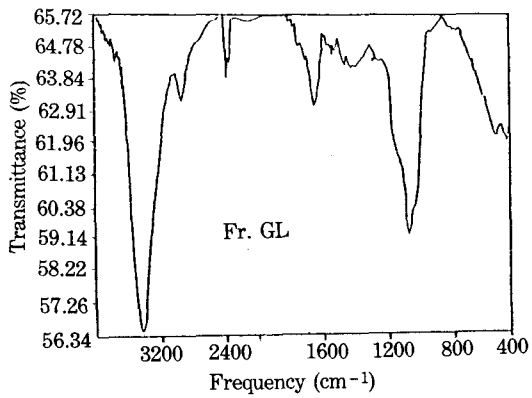


Fig. 8. IR spectrum of Fr. GL

Polysaccharide and protein contents of the five fractions were shown in Table II. The content of hexosamine in the antitumor fractions was shown in Table III. These data indicate that the major component of these fractions is a polysaccharide and that they contain 2.8-7.9% proteins with a small amount of hexosamine. The polysaccharide contents in Fractions GL and GH were increased after these fractions were purified from Fraction IN by Sepharose CL-4B gel filtration. As shown in Table IV and Fig. 9, the polysaccharide moiety of the fractions was found to be a unique heteropolymer of four different monosaccharides such as glucose, galactose, mannose, fucose or xylose, their contents being in that order. These data clearly indicate that these polysaccharides with antitumor activity are entirely different from ordinary starch or inulin which is a homopolymer of a single monosaccharide. The amino acid composition of the protein moiety in the fractions was shown in Table V and Fig. 7. The moiety consists of 17 amino acids including alanine, glycine, serine and others. It is noted that the composition is different from those of the protein moieties in the antitumor fractions that were contained in other fungi (Chang *et al.*, 1988; Chihara *et al.*, 1970; Cho *et al.*, 1988; Kim *et al.*, 1984).

Table VII. Release of SOA by induced peritoneal macrophages from normal and tumor-bearing mice

Mice	Agent	SOA release (nM/mg protein)	Ratio
Normal mice	saline	15.0±0.6*	1.00
	Fr. IN	23.1±1.2[a]	1.54
Tumor-bearing mice	saline	12.2±0.4	1.00
	Fr. IN	19.6±0.9[b]	1.60

*Mean±standard deviation, [a] $p<0.01$, [b] $p<0.05$

Table VIII. Effects of the antitumor component on hemolytic plaque forming cells in the spleen of ICR mice immunized with sheep red blood cells

	Control	Treated
Body weight (g)	*31.3± 0.6	30.1± 0.9
Spleen weight (mg)	117.5±17.9	197.5±38.3
Spleen cell count ($\times 10^7$)	20.2± 3.7	32.5± 7.3[a]
PEC/10^6 spleen cells	19.8± 7.1	128.4±26.2[b]
PFC/spleen ($\times 10^3$)	4.0± 1.6	36.9±12.2[c]

*Mean±standard deviation, [a] $p<0.05$, [b] $p<0.01$, [c] $p<0.001$

The results of the elemental analysis of the fractions were shown in Table VI. They contained carbon, oxygen, hydrogen and nitrogen, the ratio of which showed that they are carbohydrates with nitrogen compounds. The infrared spectra of the fractions were examined and found to be typical spectra of carbohydrates. The spectrum of Fraction GL was shown as an example in Fig. 8.

Effects on immune responses

The released amount of superoxide anion from the activated macrophages of the treated mice was 1.6 times higher than those of the control group. The results were summarized in Table VII, suggesting that the antitumor fraction potentiated cellular immunity against the tumor. The counts of PFC in the treated group showed about 6 times higher than those of the control group as shown in Table VIII. The results indicate that the antitumor fractions enhanced humoral immunity against the tumor.

摘 要

담자균류인 영지 *Ganoderma lucidum*의 자실체를 열수추출한 후 얻은 단백 다당체를 DEAE cellulose 이온교환수지와 Sepharose CL-4B 젤 여과법을 이용하여 분리 정제하였다. 정제한 분획들을 각각 CR, IN, IA, GL과 GH로 명명하였다. 각각의 성분을 20 mg/kg/day 용량으로 투여하였을 때 sarcoma 180 고형암에 대하여 Fr. GL이 81%의 가장 높은 종양 억제율을 나타냈으며 그 분자량은 47,000 dalton 이었다. 그 구성성분은 82% 다당류, 8% 단백질 및 0.9% hexosamine 이었다. 그 다당류를 구성하는 단당류를 분석한 결과 glucose, galactose, mannose, fucose 순으로 함유되어 있었다. 항암작용의 기전을 밝히기 위하여 마우스의 면역에 미치는 항암 성분의 영향을 연구한 결과 활성화된 마크로파지에서 분비되는 superoxide anion 양을 1.6배로, 그리고 hemolytic plaque assay에서의 용혈반 형성 세포수를 6배로 각각 증가시켰다. 이 결과로 항암작용은 고형암에 직접적으로 세포독성을 나타내는 것이 아니라, 면역세포를 활성화 혹은 강화시키는 작용임을 알 수 있다.

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References

Bradner, W.T., Clarke, D.A. and Stock, C.C. (1958): Stimulation of host defense against experimental cancer. I. zymosan and sarcoma 180 in mice. *Cancer Res.* **18:** 347.

Chang, J.B., Park, W.H., Choi, E.C. and Kim, B. K. (1988): Studies on constituents of the higher fungi of Korea (LIV). Antitumor components of *Favolus alveolarius*. *Arch. Pharm. Res.* **11:** 203.

Chaplin, M.F. and Kennedy, J.F. (1987): *Carbohydrate analysis*. IRL press, Oxford, pp.134-135.

Chihara, G., Hamuro, T., Maeda, Y., Arai, Y. and Fukuoka, F. (1970): Antitumor polysaccharide derived chemically from natural glucan (pachyman). *Nature,* **225:** 943.

Cho, H.J., Shim, M.J., Choi, E.C. and Kim, B.K. (1988): Studies on the constituents of higher fungi of Korea (LVII). Comparison of various antitumor constituents of *Coriolus versicolor*. *Kor. J. Mycol.* **16:** 162.

Cunninghm, A. (1973): Plaque assay for antibody producing cells. *Prog. Allergy,* **17:** 5.

Fudenberg, H.H. and Witten, H.D. (1981): Immunostimulation, synthetic and biological modulators of immunity. *Ann. Rev. Pharmacol. Toxicol.* **24:** 147.

Ito, M., Hidehiko, S. and Saburo, Y. (1983): Superoxide anion and hydrogen peroxide release by macrophage from treated with *Nocardia rubra* cell wall skeleton. *Gann,* **74:** 128.

Jerne, N.K., Nordin, A.A. and Henry, C. (1963): *Cell-bound antibodies,* Wistar Institute Press, Philadelphia, p.109.

Junji, H., Yukiko, Y.M. and Goro, C. (1971): Carboxymethylpachymaran, a new water soluble polysaccharide with marked antitumor activity. *Nature,* **233:** 486.

Kang, C.Y., Shim, M.J., Choi, E.C., Lee, Y.N. and Kim, B.K. (1981): Studies on antitumor components of Korean Basidiomycetes. Mycelial cul-

ture and antitumor components of *Ganoderma lucidum*. *Kor. Biochem. J.* **14:** 101.

Kato, I., Kobayashi, S., Yokokura, T. and Mutai, M. (1981): Antitumor activity of *Lactobacillus casei* in mice. *Gann,* **72:** 517.

Kikuo, N., Chikao, Y. and Kenji, T. (1975): Restoration of antibody forming capacities by PS-K in tumor bearing mice. *Gann,* **66:** 365.

Kim, B.K., Chung, H.S., Chung, K.S. and Yang, M.S. (1980): Studies on the antineoplastic components of Korean Basidiomycetes. *Kor. J. Mycol.* **8:** 107.

Kim, B.K., Shim, M.J., Kim, H.W., Woo, M.S. and Choi, E.C. (1987): Antitumor constituents of *Polyporus giganteus*. *Arch. Pharm. Res.* **10:** 179.

Kim, H.R., Shim, M.J., Kim, J.W., Kim, H.W., Lee, C.O., Choi, E.C. and Kim, B.K. (1984): Studies on constituents of the higher fungi of Korea (XLVI). Antitumor components extracted from the cultured mycelia of *Lyophyllum decastes*. *Kor. J. Pharmacogn.* **15:** 61.

Kim, J.H., Kim, H.W., Kim, J.W., Choi, E.C. and Kim, B.K. (1985): Studies on constituents of the higher fungi of Korea (LI). Antitumor components extracted from the cultured mycelia of *Volvariella bombycina*. *J. Kor. Cancer Res. Assoc.* **17:** 205.

Kim, M.J., Kim, H.W., Lee, Y.S., Shim, M.J., Choi, E.C. and Kim, B.K. (1986): Studies on safety of *Ganoderma lucidum*. *Kor. J. Mycol.* **14:** 49.

Klaus, G. (1987): *Lymphocyte,* IRL Press, Oxford, pp.119-121.

Kyoko, A., Hiroaki, N. and Hisatora, K. (1987): Potentiation of host mediated antitumor activity in mice by glucan obtained from *Grifola frondosa*. *Chem. Pharm. Bull.,* **35:** 262.

Lee, K.L., Lee, C.O., Kim, H.W., Kim, J.W., Kim, S.W., Choi, E.C. and Kim, B.K. (1985): Studies on constituents of the higher fungi of Korea (XXXVIII). Antitumor components extracted from the cultured mycelia of *Pleurotus pulmonarius*. *Kor. J. Mycol.* **13:** 11.

Lee, M.H., Kim, H.W., Shim, M.J., Choi, E.C. and Kim, B.K. (1986): Studies on constituents of higher fungi of Korea (LVI). General constituents and immunopotentiation of *Ganoderma lucidum*. *Kor. J. Mycol.,* **14:** 149.

Maeda, Y.Y. and Chihara, G. (1970): Lentinan, a new immunoaccelerator of cell mediated responses. *Nature,* **229:** 1971.

Nakahara, W., Fukuoka, F., Maeda, Y. and Aoki, K. (1964): The host mediated antitumor effect of some plant polysaccharide. *Gann,* **55:** 283.

Niels, K.J. and Albert, A. (1963): Plaque formation in agar by single antibody producing cells. *Science,* **140:** 405.

Park, H.J., Kim, H.W., Woo, M.S., Shim, M.J., Park, W.H., Choi, E.C. and Kim, B.K. (1985): Studies on constituents of the higher fungi of Korea (XLVII). Antitumor constituents of the cultured mycelia of *Agaricus campestris*. *Kor. J. Mycol.* **13:** 131.

Richard, B.J., Bernard, B.K. and Hara, P.M. (1975): The role of superoxide anion generation in phagocytic bacterial activity. *J. Clin. Investig.,* **55:** 1357.

Ringler, R.L., Byerrum, R.U., Steven, T.A., Clark, P.A. and Stock, C.C. (1957): Studies on antitumor substances produced by Basidiomycetes. *Antibiot. Chemother.,* **7:** 1.

Sabato, G.D. and Everse, J. (1988): Microassays for superoxide and hydrogen peroxide production and nitroblue tetrazolium reduction using an enzyme immunoassay microplate reader. *The Methods in Enzymology,* Academic Press, **132:** pp.407-421.

Shim, M.J., Lee, S.I. and Kim, B.K. (1978): Studies on the constituents of the higher fungi of Korea (XIV). Sterols of *Ganoderma lucidum* (Fr.) Karst. *Seoul Univ. J. Pharmaceut. Sci.* **3:** 65.

Shin, H.W., Choi, E.C., Kim, H.W. and Kim, B.K. (1985): Studies on inorganic composition and immunopotentiating activity of *Ganoderma lucidum* in Korea. *Kor. J. Pharmacogn.* **16:** 181.

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