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Antitumor Components of the Cultured Mycelia of *Ganoderma lucidum*

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Abstract—To find antitumor components in the hot water extract of the cultured mycelia of *Ganoderma lucidum*, protein-bound polysaccharides were purified and fractionated (Fr. I-V) by DEAE-cellulose ion exchange column chromatography and Sepharose CL-4B gel filtration. When a dose of 20 mg/kg/day of each was, *i.p.*, injected into ICR mice, the inhibition ratios against the solid form of sarcoma 180 were 64.2~75.8%. The antitumor component was examined for immunological activity. It increased the amount of superoxide anion released by induced macrophages in peritoneal cavity to 1.8 times and the count of hemolytic plaque-forming cells (PFC) was increased to 4.4 times as compared with those of the control group. It contained 68.6% polysaccharide which consisted of mannose, glucose, galactose, fucose and xylose and 5.1% protein consisting of 17 amino acids. The contents of hexosamine were 0.78%. The molecular weight of Fr. V that showed the highest antitumor activity was 5.8×10^4 dalton by Sepharose CL-4B gel filtration. It was named lucidan.

Keywords—*Ganoderma lucidum* • protein-bound polysaccharides • lucidan • tumor inhibition • macrophages • superoxide anion • hemolytic plaques

Antitumor polysaccharides have been isolated from natural sources such as higher plants[1], fungi[2], yeasts[3], bacteria[4] and lichens. It was Ringler who first found the antitumor activity of the basidiomycetes in 1957[5]. After that, various kinds of polysaccharides which include lentinan[6,7] obtained from *Lentinus edodes*, schizophyllan[8] from *Schizophyllum commune* and PSK[9] from *Coriolus versicolor*, were found to exhibit antitumor activity.

The antitumor polysaccharides differ greatly in their sugar composition and consequently in chemical structure, but one common point of them is their relatively high molecular weight. Although further investigation is required in clarifying mechanisms of the antitumor activity of these components, it is accepted that their action is not by direct cytotoxicity on tumor cells[7] but through host-mediated immunity. It has been reported that activated macrophages, NK cells, cytotoxic T cells and killer T cells usually play important roles in tumor immunity[10,11].

Fruiting bodies of a fungus, *Ganoderma lucidum* are well known as a crude drug distributed in the oriental countries and used to cure various human diseases, such as chronic hepatitis, nephritis, gastric ulcer, hypertension, arthritis, neurasthenia, insomnia, bronchitis, asthma and poisoning. But scientific studies on *Ganoderma*

lucidum were not published until 1970's. So far various researches have been carried out on pharmacologically effective components of the fruiting body and the cultured mycelia of *Ganoderma lucidum* and it has been suggested that they were effective on hypercholesteremia, essential hypertension[16], hypoglycemia[17], disseminated intravascular coaggulation[18], histamine release[19], and platelet aggregation[20].

Several β-glucans, appertaining to the same molecular species but having different degrees of branching were isolated from the water and alkali extracts of the fruiting body and the culture filtrate of *Ganoderma lucidum*[21], and systematic fractionation was attempted to obtain their relationship to the antitumor activities[22~27].

In the present study, protein-bound polysaccharides were extracted with hot water from the cultured mycelia of *Ganoderma lucidum* in Korea and examined for antitumor activity in mice. The antitumor components were purified by ion exchange chromatography and gel filtration method. Their chemical composition and molecular weight (MW) were elucidated by several analyses. In addition, their effects on immune responses in mice were examined to explain mechanisms of the antitumor activity.

Experimental Methods

Strain and Culture

The strain of *Ganoderma lucidum* (Fr.) Karsten DMC-9 (the family *Ganodermataceae*) used in this work was kindly provided by Agricultural Science Institute at Suwon, Gyeong-Gi Province, Korea. The mycelia of *Ganoderma lucidum* were cultured on a cellophane-laid fresh PDA plate and inoculated into 100 ml of the culture medium. For main culture, the mycelial pellets from the second culture were aseptically homogenized, transferred into 500 ml of the culture medium (inoculum size: 20% v/v) and cultured for 10 days.

Extraction and Separation of Fungal Metabolites

The mycelia from 38 liters of the culture broth were extracted twice with distilled water (d.w.) at 95°C for 6 hr. The filtrates were concentrated and three volumes of 95% cold ethanol were added. The resulting precipitates were dissolved in d.w., dialyzed and lyophylized to obtain Fraction I. Fraction I (4.38 g) was applied to the DEAE-cellulose(Cl^- form, Sigma Chem. Co., U.S.A.) column. It was eluted with d.w. (pH 7.2). Against each fraction, optical densities were measured at 625 nm (anthrone test), 595 nm (Bradford assay) and 540 nm (Lowry-Folin method). The anthrone-positive fraction was designated Fraction II. The adsorbate on DEAE-cellulose was eluted with 2M NaCl. A brownish powder was obtained with a yield of 1.60 g and designated Fraction III. A solution of Fraction II (500 mg) in 0.01M sodium phosphate buffer (pH 6.8) was applied to the pre-swollen Sepharose CL-4B (Pharmacia Co., Sweden) column. It was eluted with the same buffer and optical densities were measured at 625 nm(anthrone test) and 595 nm(Bradford assay) against each fraction. The anthrone-positive fractions were designated Fraction IV (6 mg) and Fraction V (200 mg), respectively.

Antitumor Test

Male ICR mice(20~25 g) and sarcoma 180 tumor cells were used for antitumor test *in vivo*. Fractions I, II, III and V were *i.p.* administered (20 mg/kg/day) into the mice on 3rd day after the tumor inoculation and continued once a day for consecutive ten days. Krestin was used as positive standard and physiological saline as negative control. On the 25th day after the tumor inoculation, the solid tumors were excised and weighed.

Effects of Antitumor Components on Immune Responses

Effects on macrophage(Mφ) activation: Twelve mice were divided into 4 groups, two of which were prepared for control groups and the other two for treated groups. Tumor cells(2×10^6 cells/ml) were inoculated *s.c.* into the left groins of ICR mice. For the two treated groups, 0.1 ml of Fraction II solution in physiological saline was injected *i.p.* to ICR mice at a dose of 40 mg/kg/day, for 5 consecutive days from the day when the tumor cells were inoculated. On the fifth day after completion of sample administration, peritoneal Mø were obtained and washed with HBSS. The cells were cultured in a 5% CO_2 incubator at 37°C for 2 hr on the 24-well plate (Falcon Co., U.S.A.). The non-adherent cells were removed by washing 3 times with HBSS.

To Mø which was fixed to the plate, 1.5 ml of PBS containing 10 mM glucose, 80 μM ferricytochrome C (Sigma Chem. Co., U.S.A.) and 0.2 mg/ml of opsonized zymosan(Sigma Chem. Co., U.S.A.) were added and the plate was incubated for 90 min at 37°C. After incubation, the culture was centrifugated and its supernatant was transferred into an ice-chilled test tube and optical density (OD) was measured at 550 nm. On the other hand, 1 ml of 0.5% sodium dodecyl sulfate soln was added to the precipitated cells, then after leaving 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 the absorbance at 550 nm and free superoxide anion(SOA) per unit protein was calculated[14].

$\Delta E_{550}=2.1\times10^4 M^{-1}cm^{-1}$

SOA nmol/well=OD at 550 nm×15.87

SOA nmol/mg protein=(OD at 550 nm×15.87)/Mø amount (mg)

Effects on hemolytic plaque forming cells (PFC)[15]: Twelve mice were divided into two groups. For a treated group, 0.1 ml of Fraction II (40 mg/kg/day) soln was *i.p.* injected for five consecutive days. Physiological saline was used as control. On the 7th day after completion of injection, the mice were immunized by *i.p.* injection of 1×10^7 cells/0.1ml of sheep red blood cells. On the 5th day after immunization, the spleens were excised and homogenized with ice-cold HBSS and centrifugated. After hemolysis with 0.83% NH_4Cl, the cells were washed three times and centrifugated and resuspended in ice-cold HBSS. The spleen cells were counted directly with a hemacytometer and then diuted to 1×10^7 cells/ml. Splenocyte(150 μl), 50% SRBC(25 μl), 0.05% DEAE dextran-agar and guinea pig complement(150 μl) were mixed and placed on the culture plates and overlayed with a microscopic cover glass and allowed to solidify. The agar plates were incubated for 3 hr at 37°C. The number of hemolytic plaques was counted[15].

Chemical, Physical and Instrumental Analyses

Elemental composition was determined by Perkin-Elmer elemental analyzer. Total polysaccharide contents were quantitatively determined by anthrone test. Bradford assay[28] was adopted for total protein contents. Free hexosamines and free N-acetylhexosamines in each fraction were quantitatively determined by Elson-Morgan method[32]. For monosaccharide analysis, each fraction and standard monosaccharides were methanolized at 80±5°C for 20 hr in anhydrous 3% HCl-MeOH. The methanolysates were silated and analyzed by Shimadzu gas chromatography RLA. To analyze amino acids of the protein moiety in the antitumor components, each fraction was hydrolyzed at 100±5°C for 24 hr in 6N HCl and analyzed by Hitachi automatic amino acid analyzer 835. Sepharose CL-4B gel filtration was used for molecular weight(MW) determination as well as in purification of neutral polysaccharides. Standard polysaccharides were dextran(Pharmacia Co., $MW=2\times10^6$, T-2000), dextran(Sigma Chem. Co., $MW=4.8\times10^5$, T-480) and dextran (Nakarai Chem. Ltd., $MW=6\times10^4$, T-60).

MW of Fraction V was determined graphically.[33] For infrared(IR) spectroscopy, each sample was analyzed by KBr disc method with Perkin-Elmer IR 20 analyzer.

Results

Purification of Polysaccharides of *Ganoderma lucidum*

The cultured mycelia of *Ganoderma lucidum* were extracted with d.w., and 6.0 g of dark brown powder(Fr. I) was obtained from 38 liters of the culture broth. When Fr. I(4.38 g) was applied to the DEAE-cellulose ion exchange column, the neutral polysaccharide fraction eluted by d.w. was 1.31 g of white powder(Fr. II) and the acidic polysaccharide fraction eluted by 2M NaCl(at high ionic strength) was 1.60 g of brown powder (Fr. III). The elution profile was shown in Fig. 1. Fraction II(500 mg) was further purified by using Sepharose CL-4B gel filtration column and eluted by 0.01M sodium phosphate buffer in pH 6.8. The first fraction separated(Fr. IV) was extremely small amount (6 mg) of a higher MW fraction and the second fraction(Fr. V) was 200 mg of a white powder with lower MW. The elution profile was shown in Fig. 2.

Antitumor Activity

Tumor inhibition ratios of each fraction and

Table I. Antitumor activities of the protein-bound polysaccharides obtained from *Ganoderma lucidum*

Group	Dose*	Average tumor weight (g)**	I.R.(%) ***	Complete regression
Control	saline	5.78±1.46	—	0/8****
Krestin	20	2.44±0.92	57.9	0/8
Fr. I	20	1.98±1.51	65.8	1/8
Fr. II	20	1.40±0.66	75.8	1/8
Fr. III	20	2.06±1.23	64.4	1/9
Control	saline	5.78±1.20	—	0/10
Krestin	20	3.16±1.91	45.3	0/10
Fr. II	20	2.07±1.47	64.2	1/10
Fr. V	20	1.59±0.84	72.5	0/10

$p<0.01$

* mg/kg/day, *i.p.*

** Mean ± standard error

*** $I.R. = \frac{\text{Control tumor weight} - \text{Treated tumor weight}}{\text{Control tumor weight}} \times 100$

**** Number of ICR mice used

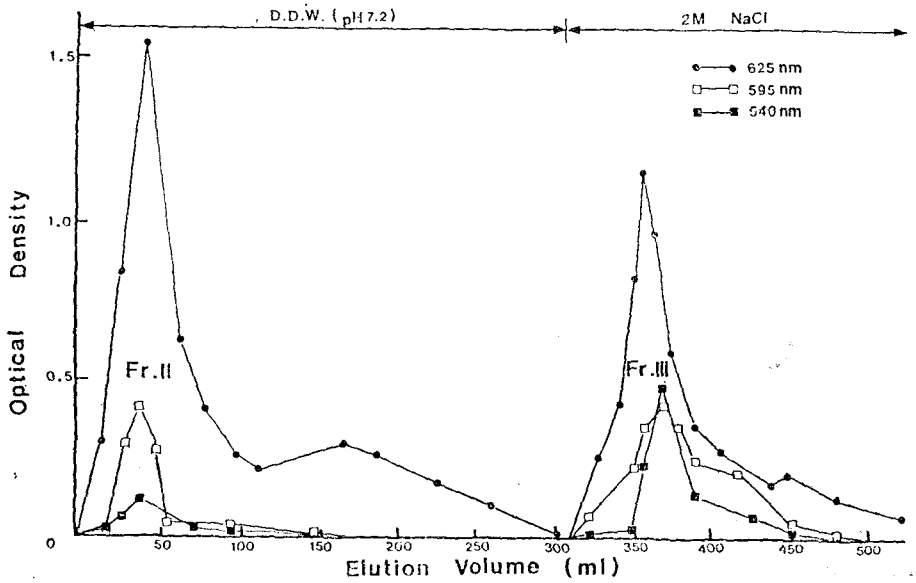

Fig. 1. The elution profile of Fraction I obtained from the mycelia of *Ganoderma lucidum* by DEAE-cellulose ion-exchange chromatography

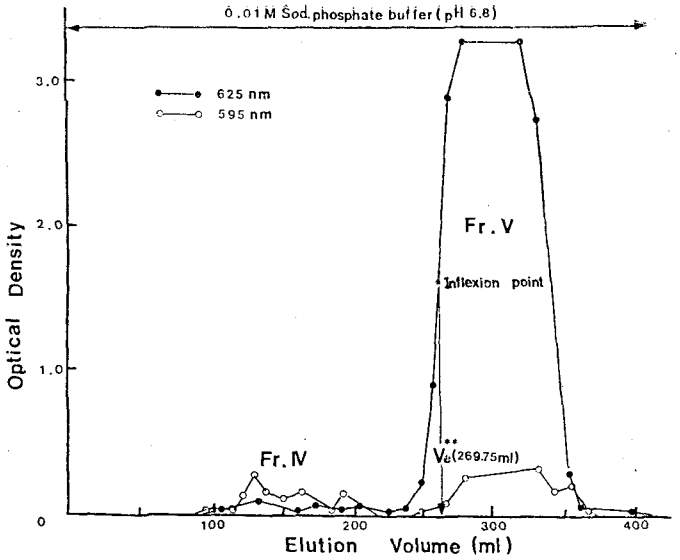

Fig. 2. The elution profile of Fraction II obtained from the mycelia of *Ganoderma lucidum* by Sepharose CL-4B gel filtration chromatography
* Half height of the rising part of elution peak
** Elution volume for the calculation of molecular weight of Fraction V

the standard agent Krestin against sarcoma 180 cells in mice were shown in Table I. All the fractions suppressed the growth of tumor, *i.e.*, Krestin, Fr. I, Fr. II, Fr. III and Fr. V showed antitumor activities of 57.9, 65.8, 75.8(or 64.2), 64.4 and 72.5%, respectively. Among them, complete regressions were appeared in the Fr. I(1/8), Fr. II(1/8 or 1/10) and Fr. III(1/9).

Table II. Effects of the antitumor component on the release of SOA by induced macrophages(Mϕ) from normal and tumor-bearing mice

Group		Mϕ contents (mg/ml)	SOA released (nmol/mg protein)	Ratio(t/c)
Normal	Control	0.133±0.034*	14.797±0.833	1.81
	Treated	0.193±0.057	26.725±1.233	
Tumor	Control	0.101±0.030	15.713±3.300	1.47
	Treated	0.142±0.065	23.134±1.676	

Table III. Effects of the antitumor component on the hemolytic plaque forming cells(PEC) in the spleen of ICR mice immunized with SRBC

	Control group	Treated group
Body weight(g)	20.38± 1.36*	20.81± 0.81
Spleen weight(mg)	188.00±10.00	162.00±20.00
Spleen cell count ($\times 10^7$)	18.52± 3.93	20.76± 4.37
PFC/10^6 spleen cells	29.51± 6.94	88.04±15.71
PFC/spleen($\times 10^3$)	5.27± 1.30	23.19± 3.51

* Mean ± standard error

Effects of Antitumor Component on Immune Responses

Effects on macrophage activation: The amounts of activated macrophages and free SOA were the maximum in normal mice treated with Fraction II. Compared with each control group, the treated groups were 1.45 times higher in their peritoneal macrophage amount. SOA release by peritoneal macrophages was also increased to 1.81 times in the treated normal mice. The results were shown in Table II. These results indicate that the fraction augmented the activity of macrophages.

Effects on hemolytic plaque forming cells (PFC): The PFC: The PFC counts of the treated group showed about 4.4 times greater than those of the control group. Average weight of the spleens in the treated group was increased to 17.39%. The results were summarized in Table III. These data indicate that the fraction enhanced the activity of B lymphocytes.

Chemical, Physical and Instrumental Analyses

Chemical analyses: The elemental composition of each fraction was carbon, oxygen, hydrogen and nitrogen. Their ratios were shown in Table IV, indicating the usual ratios of carbohydrates. The contents of the polysaccharide and the protein in each fraction were shown in Table V. Average polysaccharide contents were 68.6%.

Table IV. Elemental compositions of the antitumor components of *Ganoderma lucidum*

Fraction	C	H	O	N
I	6.00*	9.72	4.92	0.72
II	6.00	10.06	6.70	0.42
III	6.00	10.08	6.83	0.90
IV	6.00	10.24	6.43	0.72
V	6.00	10.38	5.51	0.30

* Expressed as the mole ratio.

Bradford assay was compared with Lowry test in protein quantification. When the protein contents of two peak fractions at 625 nm in ion-exchange effluents were calculated by the two tests, those of d.w.-eluted peak fraction were the same in the two tests. While those of 2M NaCl-eluted peak fraction were about six times more in Lowry test than in Bradford assay (Table VI). To examine effects of the brown pigments of Fr. III on this difference, absorbances of Fr. III (10 mg/2 ml) were scanned at

Table VI. Comparison of Bradford assay with Lowry test at two elution peak points in ion-exchange chromatography

Eluent	Protein contents by		Ratio
	Lowry test (540 nm)	Bradford assay (595 nm)	(L/B)
D.D.W.	30.0± 3.7**	24.0±3.2	1.3
2M NaCl	155.3±18.6	24.9±2.7	6.2

** Mean ± SE

Table V. Polysaccharide and protein contents of the fractions

Fraction	Polysaccharide contents from calibration curve of				Protein contents
	Mix. 1[a]	Mix. 2[b]	Man.[c]	Glu.[d]	
I	62.1±7.5[e]	—	65.7±4.2	—	9.3±0.05
II	66.6±9.8	—	70.4±4.7	—	1.8±0.11
III	—	31.3±7.5	—	31.4±2.0	7.3±0.08
V	77.2±3.4	—	81.5±6.6	—	2.1±0.04

a: Mixture of glucose (47%), fucose (30%), mannose (12%) and galactose (11%).
b: Mixture of glucose (20%), fucose (10%), mannose (36%), galactose (28%) and xylose (6%).
c: Mannose
d: Glucose
e: Mean ± SE (weight percent)

Table VII. The contents of free hexosamine and N-acetylhexosamine of each fraction

Fraction	Total hexosamine(%, w/w)
I	0.92±0.14*
II	0.51±0.03
III	0.66±0.02
V	1.04±0.29

* Mean ± standard error

540 mn and 595 nm, and then calculated in terms of protein contents. The results also showed about six times greater at 540 nm than at 595 nm (data not shown). From the results of these comparisons, Bradford assay was selected for determination of total protein contents of each fraction and average protein contents were 5.1%. Free hexosamines and free N-acetylhexosamines in each fraction were quantitatively determined and average contents of them were 0.78% (Table VII). As shown in Table VIII, monosaccharide subunits of the polysaccharide moiety in each fraction were mannose, glucose, galactose, fucose

Table VIII. Monosaccharide contents of the polysaccharide moiety of the antitumor components by GLC analysis

Fraction	Mannose	Glucose	Galactose	Fucose	Xylose
I	41.14*	15.47	26.45	12.15	4.79
II	29.90	27.83	27.89	6.44	7.93
III	11.72	47.45	10.57	30.25	N.D.**
V	35.87	17.50	31.01	11.41	4.21

* Expressed as the area percentage.
** Not detected.

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

Amino acids	Retention time(min)	Fr. I	Fr. II	Fr. III	Fr. V
L-Aspartic acid	8.81	13.47*	9.10	14.75	8.82
L-Threonine	9.66	8.85	13.12	8.24	11.07
L-Serine	10.41	9.45	12.65	9.32	12.35
L-Glutamic acid	11.40	12.87	7.66	14.63	9.98
L-Proline	12.37	8.56	7.16	9.89	6.52
Glycine	16.17	12.31	12.57	10.94	8.82
L-Alanine	17.53	5.72	7.31	5.77	7.46
L-Cysteine	19.33	0.84	0.44	0.61	0.53
L-Valine	22.60	4.65	5.79	5.88	6.18
L-Methionine	24.01	1.17	N.D.**	0.73	2.33
L-Isoleucine	26.18	2.87	2.61	2.82	3.92
L-Leucine	27.48	4.66	4.66	5.01	5.71
L-Tyrosine	29.46	1.96	1.93	1.91	1.57
L-Phenylalanine	30.86	4.08	3.72	2.90	6.73
L-Lysine	35.53	3.87	5.13	4.14	4.80
L-Histidine	39.57	0.87	4.15	0.84	1.20
L-Arginine	46.72	1.61	2.01	1.61	2.03

* Expressed as the mole percentage.
** Not detected.

and xylose. The compositions of 17 amino acids contained in the protein moiety were shown in Table IX.

Determination of MW: MW of Fraction V was determined by Sepharose CL-4B gel filtration chromatography. The elution volume of three standard dextrans(MW: 6×10^4, 4.8×10^5 and 2×10^6) and standard calibration curve was obtained from the gel filtration. From this curve, MW of Fraction V was found to be about 5.8×10^4 (Fig. 3).

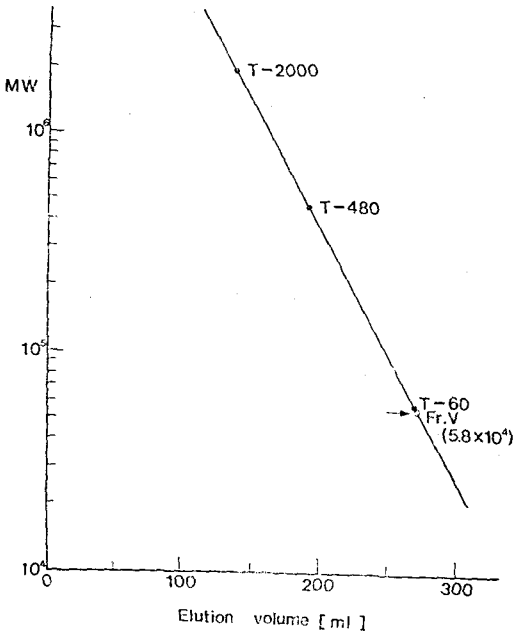

Fig. 3. Standard calibration curve for the determination of molecular weight of Fraction V
The elution volume was plotted against the logarithm of molecular weight.

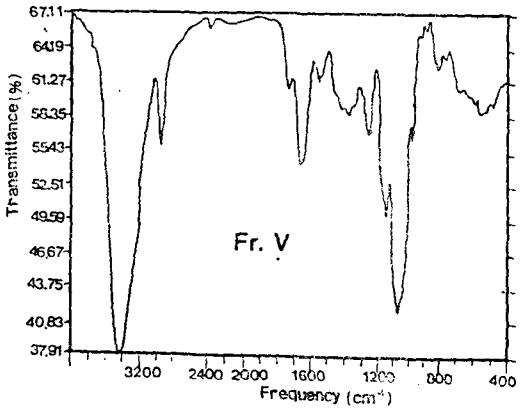

Fig. 4. IR spectrum of Fraction V

IR spectroscopy: IR spectrum of Fraction V was shown in Fig. 4. It had the typical characteristics of carbohydrates.

Discussion

The protein-bound polysaccharides obtained from the cultured mycelia of *Genoderma lucidum* showed an antitumor activity against the solid form of sarcoma 180, when they were injected intraperitoneally into ICR mice. Tumor inhibition ratio of each fraction was higher than that of Krestin used as standard antitumor agent and the purified Fraction V exhibited 72.5% inhibition ratio. It was found that the more it was purified, the more potent its antitumor activity was.

The examination of the effects of Fraction II on immune responses in mice showed that the antitumor component activated the peritoneal macrophages, the first major cell type to react with an antigen or tumor cells in most immune responses, and that the release of superoxide anions from the induced macrophages was enhanced. In the normal mice, macrophages were more activated than in the tumor-bearing mice. It was also found that this component increased the spleen weight and the number of the plaque-forming cells which correspond to that of the antibody-forming B cells in the spleen.

The elemental analysis of the antitumor fractions showed that the average ratio of C : H: O : N in each fraction was 6.00 : 10.10 : 6.08: 0.61. The data indicate that these components were composed of a carbohydrate and a small amount of protein. Fraction I contained 62.1% polysaccharide and 5.1% protein, and Fraction V 77.2% polysaccharide and 2.1% protein. It appears that the protein and the polysaccharide were bound since the protein moiety was not

completely removed during the purification. The detection of 0.78% hexosamine in the antitumor components suggests that the protein was bound with the polysaccharide. The main monosaccharides constituting the polysaccharides were mannose, glucose, galactose, fucose and xylose. And the protein moiety contained 17 amino acids, especially aspartic acid, glutamic acid and glycine. The IR spectra of the fractions showed the typical characteristics of polysaccharides, *i.e.*, O-H stretching frequency at 3300~3400 cm^{-1}, C-H stretching frequency at 2900 cm^{-1}, C-O stretching frequency at 1630 cm^{-1} and C-H and C-O bending frequency at 1000~1100 cm^{-1}.

For total protein contents, Bradford assay was adopted and carried out. It had several advantages as compared with Lowry test which had been ordinarily used. To examine which method is more accurate in protein assay of the extracts from the mycelia of *Ganoderma lucidum*, Bradford assay was compared with Lowry test. The protein contents of two elution peak fractions which had showed the highest absorbances at 625 nm in ion-exchange effluents were calculated by the two tests and compared. And to detect the effects of pigments on the two tests, optical densities of the pigments (Fraction III, 10 mg/2 ml) were scanned at 595 nm and 540 nm, and then they were calculated in terms of protein contents. Two standard calibration curves were made with Lowry reagent and Bradford reagent using bovine serum albumin. Since Bradford method was not affected by the fungal brown pigments in protein assay, it was found that this method was more accurate than Lowry-Folin method.

Conclusion

The mycelia of *Ganoderma lucidum* were grown by submerged culture in artificial media. The high molecular weight components were extracted from the harvested mycelia and further purified, being separated into five fractions. They inhibited the growth of the solid form of sarcoma 180 in mice. Fraction V showed an inhibition ratio of 72.5%. Fraction II suppressed the tumor growth by enhancing both humoral and cellular immunities of the host. Fraction V was found to be a protein-bound polysaccharide, consisting of 77.2% polysaccharide and 2.1% protein and 1.1% hexosamine. The polysaccharide moiety of the Fraction V consisted of 35.9% mannose, 31.0% galactose, 17.5% glucose, 11.4% fucose and 4.2% xylose, the protein moiety consisting of 17 amino acids. Fraction V had molecular weight of 5.8×10^4 Da and was named lucidan.

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