갓버섯의 抗癌 成分

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Antitumor Components of the Cultured Mycelia of *Lepiota procera*

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ABSTRACT—To find antitumor components in the cultured mycelia of *Lepiota procera*, the protein-polysaccharide obtained from the mycelia was subjected to DEAE – Sephadex A-50 column chromatography and Sepharose-4B gel filtration. Of the fractions, the purified Fraction C_1 was named lepiotan and examined for antitumor activity against the solid form of sarcoma 180 in ICR mice. The inhibition ratio of lepiotan was 64% at the dose of 10 mg/kg/day for 10days. The chemical analysis of lepiotan showed 60% polysaccharide and 21% protein. The polysaccharide moiety was found to be a heteromannoglucan which consisted of 46.3% glucose, 40.2% mannose and 11.0% fucose. When the antitumor component, Fraction A, was examined for immunopotentiation activity, it was found to increase the number of plaques in hemolytic plaque assay and to restore the immunity in the tumor-bearing mice up to 89.7% of the normal level. Also the antitumor acitivity was suppressed by the treatment with carrageenan, an anti-macrophage agent. These results indicate that the antitumor activity was exerted through immunopotentiation, but not through direct cytotoxicity against the tumor.

Keywords □ ***Lepiota procera*, Basidiomycetes, Protein-polysaccharide, Lepiotan, Antitumor components.**

Many antitumor preparations were obtained from a variety of natural sources such as higher plants[1], fungi[2], yeasts[3], bacteria[4] and lichens. It was Ringler[5] who first found the antitumor activity of the basidiomycetes in 1957. Various kinds of basidiomycetes preparations which include: lentinan[6, 7], a high molecular weight β-1, 3-glucan obtained from *Lentinus edodes* fruiting bodies; schizophyllan[8], a high molecular weight β-1, 3: 1,6 glucan prepared from *Schizophyllum commune* culture filtrates; and PS-K[9], a peptide containing β-1, 4: 1,3 or β-1, 4:1, 6-glucan extracted from *Coriolus versicolor*[10] culture mycelia, were shown to exhibit antitumor activity. Although the mechanism of the antitumor activity of these components has not been completely elucidated, it is suggested that the action is not direct cytotoxicity on tumor cells[7], but by host-mediated immunity.

In recent years, our laboratory has been reporting on the antitumor components from the carpophores and cultured mycelia of Korean basidiomycetes to find antitumor components with

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lower toxicity[11-19]. *Lepiota procera* was new to Korea and it was reported that this fungus was antiboitic against a fungus cultivated by gardening ants[20]. Therefore attempts were made to investigate components of this fungus.

In the present study, the cultured mycelia of this fungus were extracted with hot water and the extract was examined for antitumor activity in mice. The antitumor components were purified by ion exchange chromatography and gel filtration method. It was also analyzed to find its chemical composition. In addition, to study modes of the action, its effects on immune responses were examined. It was noted that the protein-polysaccharide fraction affected antibody production and delayed-type hypersensitivity reaction. Its antitumor activity was also suppressed by treatment with carageenan, an anti-macrophage agent.

MATERIALS AND METHODS

Strain and Culture Condition—The strain of *Lepiota procera* (the family Agaricaceae) used in this study was kindly provided by Agricultural Science Institute at Suweon, Gyeong-Gi Province, Korea. The mycelia of *L. procera* were aseptically tranferred into a fresh PDA slant and cultured for seven days at 27±1°C. The fully grown mycelia were separated aseptically and homogenized with a small volume of the culture medium for 10 seconds in a microblender. It was inoculated into 10 m*l* of the culture medium in a 50- m*l* flask and incubated for 10-15days in an orbital shaking incubator at 27±1°C, 180 rpm. Then, the mycelial pellets were aseptically homogenized for 10seconds and inoculated into a 500- m*l* flask containing 100 m*l* of the culture medium. Incubation was carried out for nine days under the same condition of the first culture. The mycelial pellets from the second culture were aseptically homogenized for 10 seconds and transferred into 500 m*l* of the culture medium in a two-liter flask (inoculum size: 4 v/v %) and cultured for nine days under the same condition as previously described (Fig. 1).

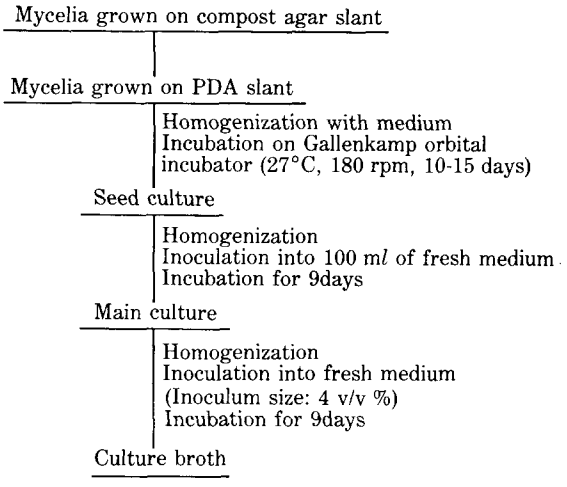

Fig. 1. Culture method of *Lepiota procera*.

Extraction and Separation of Antitumor Components—The mycelia from 25 *l* of the culture broth were filtered and washed twice with distilled water (d.w.). The obtained mycelia were homogenized and extracted with d.w. in an autoclave (121°C, 2atms) for 30 min. This process was repeated. After filtration, the filtrates were concentrated under vaccum and three volumes of 95% ethanol were added to the concentrate. In order to complete the precipitation, the mixture was allowed to stand at 0-4°C overnight. The precipitates were collected by centrifugation at 10,000×g for 30 min, dissolved in d.w. and dialyzed at 0-4°C for seven days using Visking tube (36/22). Water-insoluble substances were removed by filtration and water-soluble substances were obtained as a dark brownish powder with a yield of 4.25g. It was designated Fraction A. The filtrates obtained from the culture broth were also treated as the above process. It was designated Fraction E (0.78g/*l*)(Fig. 2). In order to remove the protein portion, the protein-polysaccharide (1g) was dissolved in 50 m*l* of d.w. and pronase was added to the solution (100 mg, Yung Jin Pharm. Inc. Co., Ltd). The mixture was shaked for 45 min at 37°C and 180 rpm and treated by Sevag method. It was designated Fraction B (12 mg)(Fig. 3).

DEAE-Sephadex A-50 (borate form) was packed into a column (4.4×12 cm) with 0.01 M

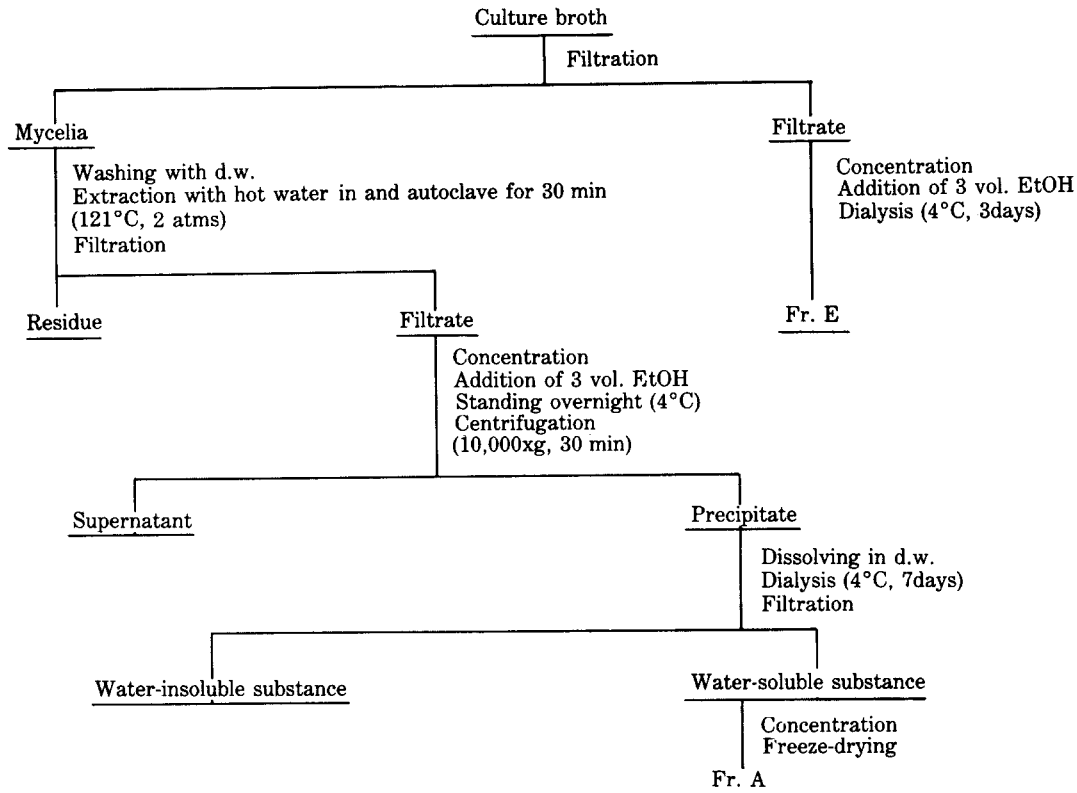

Fig. 2. Extraction and separation of antitumor components from the cultured mycelia of *Lepiota procera*.

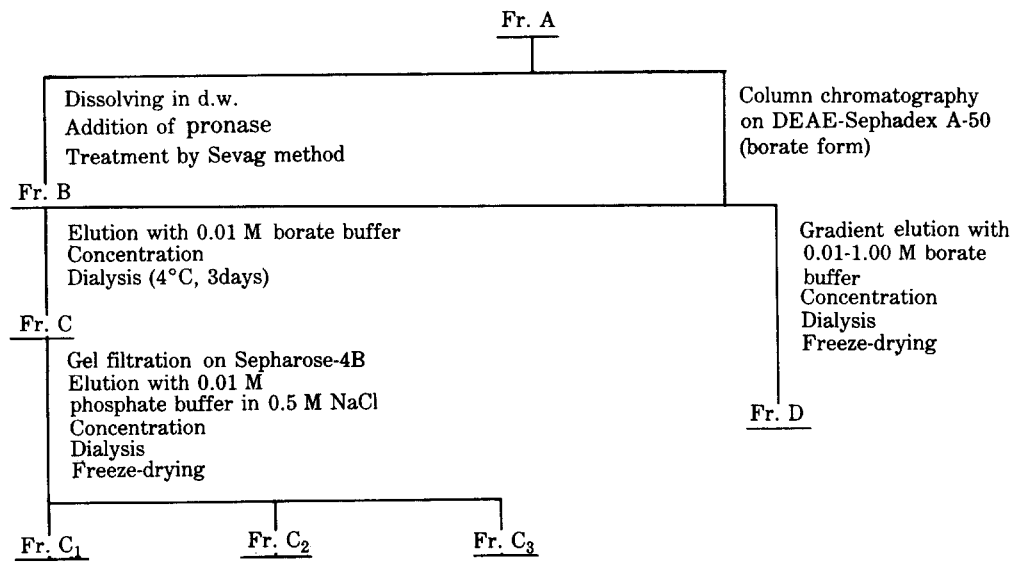

Fig. 3. Purification of antitumor components of *Lepiota procera*.

borate buffer (pH 8.7). A solution of Fraction A (2.1 g) in eluant (5 m*l*) was applied to the column. Then, it was eluted with 0.01 M borate buffer (pH 8.7) at a flow rate of 12 m*l*/hr. Against each fractions, absorbance at 625 nm was measured. The anthrone-positive fractions were concentrated, dialyzed and lyophilized. Thus, the unadsorbate on DEAE-Sephadex was obtained as a white powder with a yield of 570 mg. It was designated Fraction C. The adsorbate on DEAE-Sephadex was eluted with 0.01-1.00 M linear gradient borate buffer (pH 8.7). The pale brownish powder which was designated Fraction D was obtained (211 mg) (Fig. 3).

Preswollen Sepharose—4B (Pharmacia Co.) was packed into a column (2.4×30 cm) with 0.01 M phosphate buffer in 0.5 M NaCl (pH 7.0). A solution of Fraction C (500 mg) in eluant was applied to the column. Then, it was eluted with 0.01 M phosphate buffer (pH 7.0) containing 0.5 M NaCl at a flowrate of 10 m*l* per 30 min. Absorbance at 625 nm (anthrone test) and 540 nm (Lowry-Folin test) was measured. Anthrone-positive fractions were collected, concentrated, dialyzed and lyophilized. The white powders which were designated Frs. C_1 (62 mg), C_2 (74 mg), and C_3 (12 mg) were obtained (Fig. 3)

Assay of Antitumor Test—Male ICR mice (18-20g) were supplied from the Experimental Animal Farm of Seoul National University. Each forty milligrams of Frs. A, C, C_1, and E were dissolved in 10 m*l* of saline for a dose 20 mg/kg. Also, each twenty milligrams of Frs. B and C_1 were dissolved in 10 m*l* of saline for a dose 10 mg/kg. Physiological saline was used for control. Sarcoma 180 cells(1×10^6 cell/0.1 m*l*/mouse) were implanted subcutaneously into the right-flank of ICR mice. Seven or eight mice were used for each group. The test solutions of Frs. A, B, C, C_1, C_2 and E were respectively injected intraperitoneally every day for 10 days at the doses of 20, 10, 20, 10, 20 and 20 mg/kg/day, starting on the third day after the tumor implantation. To the control group, saline was injected. Tumor weights were measured on the 30th day after

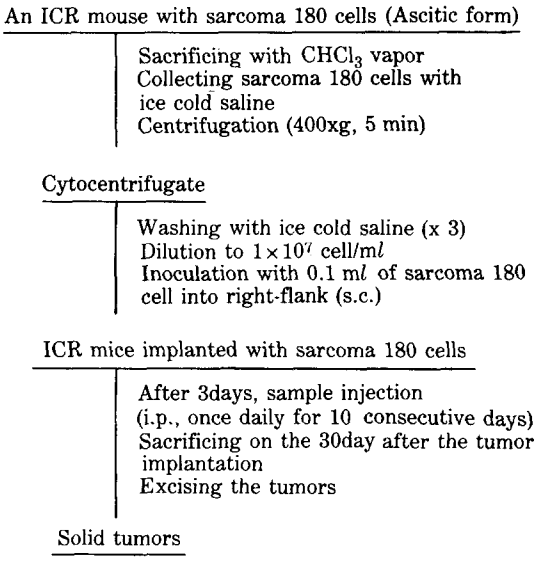

Fig. 4. Antitumor test procedure *in vivo*.

tumor implantation and tumor inhibition ratio was calculated as follows (Fig. 4).

$$\text{Tumor Inhibition Ratio (\%)} = \frac{C_W - T_W}{C_W} \times 100$$

Where, T_W: Average tumor weight of the treated group

C_W: Average tumor weight of the control group

To investigate life span, sarcoma 180 cells (1×10^6 cell/0.1 m*l*/mouse) were implanted into the peritoneal cavity of male ICR mice weighing about 25g. Twenty mice were divided into two groups: one control group and the other treated group. The test solution of Fr. C_1 was injected intraperitoneally for 10 consecutive days at the dose 10 mg/kg/day, starting on the first day after the tumor implantation and physiological saline was used for control. The survival of the mice was observed for 34 days.

Survival rate (T/C, %)

$$= \frac{\text{Mean survival days of treated mice}}{\text{Mean survival days of control mice}} \times 100$$

Chemical analysis—Total polysaccharide contents were quantitatively determined by anthrone test using D-glucose as standard. After anthrone test, the polysaccharide content was calculated from U.V. absorbance at 625 nm.

Total protein contents were determined using bovine serum albumin(Sigma,USA) as a standard protein by Lowry-Folin test U.V. absorbance at 540 nm.

For monosaccharide analysis, each fraction (10 mg) and each standard monosaccharide (5 mg) was respectively dissolved in 3% HCl-methanol (2 m*l*) and methanolysed at 80±5°C for 20 hours in a capped test tube filled with nitrogen gas. The methanolysate was filtered, evaporated and dissolved in pyridine (1 m*l*). Trimethylsilylation was carried out with 0.2 m*l* of hexamethyldisilazane and 0.1 m*l* trimethylchlorosilane. Gas chromatography showed several monosaccharides of the fractions by comparison with retention times of standard monosaccharides. The content of each monosaccharide was calculated from the chromatograms by measuring the peak area.

For infrared spectra, each fraction (1 mg) was analyzed by KBr disc method.

Effects on hemolytic plaque-forming cells- Ten mice (Male ICR mice, 18-20g, supplied from the Experimental Animal Farm of Seoul National university) were divided into two groups. For a treated group, 40 mg of Fraction A was dissolved in five m*l* of saline and 0.1 m*l* of this solution was injected intraperitoneally once a day for five consecutive days. Physiological saline was used for control group. On the seventh day after the last sample administration, the mice were immunized by intraperitoneal injection of 1×10^7 cells of SRBC. After five days of immunization, the mice were sacrificed and the spleens were dissected. The spleens were homogenized with ice-cold BSS and centrifugated 400×g for five min. Cytocentrifugates were collected and hemolyzed with 0.83 % NH_4Cl solution at 37°C for five min. After hemolysis, the suspension was centrifugated under the same condition and the cytocentrifugates were

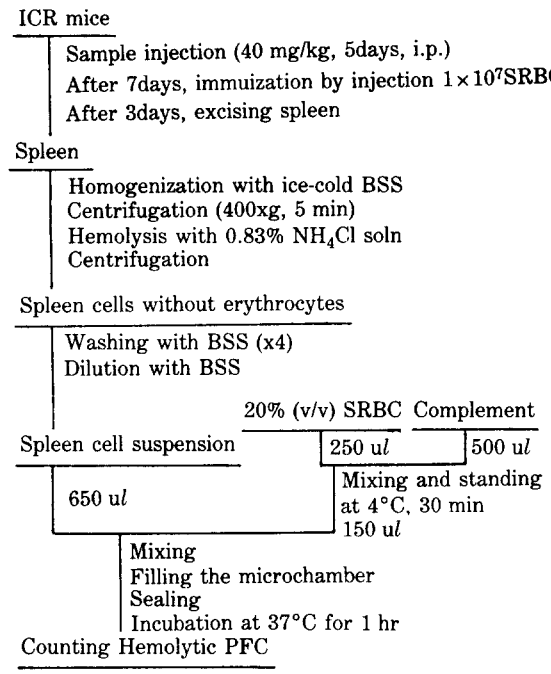

Fig. 5. Assay procedure of hemolytic plaque-forming cells.

resuspended in ice-cold BSS. The spleen cells were counted directly by a hemacytometer.

In the mean time sheep red blood cells were centrifugated and resuspended in physiological saline to adjust the concentration into 20 v/v%. And then, 500 u*l* of 20 v/v% SRBC solution and 650 u*l* of spleen cell suspension, and 100 u*l* of this mixture was injected into the microchamber. After sealing the microchamber with vaselin and wax (1:1), incubation was carried out at 37°C for an hour and the numbers of hemolytic plaques were counted (Fig. 5).

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

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

$$a = \frac{\text{600 (volume of spleen cells suspension)}}{\text{800 (volume of incubation mixture)}}$$

N: number of plaques observed in one microchamber
c: count of spleen cells in one m*l* of spleen cell

suspension

V_m: volume of incubation mixtre filled into one microchamber (m*l*)

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

Effect on delayed-type hypersensitivity—Twenty male ICR mice (5-6 weeks age) were divided into four groups: Normal mice group (N), Fraction A treated group (A), Tumor bearing group (T), Tumor bearing and Fraction A treated group (TA). For TA group, sarcoma 180 cells (1×10^6 cell/mouse) were inoculated subcutaneously into the right-flank of ICR mice and after three days, forty milligrams of Fraction A were dissolved in 5 m*l* of physiological saline and 0.1 m*l* of this solution was injected to ICR mice once a day for consecutive five days (i.p.). For A group, sample administration was conducted in the same method as TA group. Physiological saline was used for N group and T group at the same time. On the last sample injection, the mice were immunized by injection of 10^8 SRBC in 0.05 m*l* of saline into the right hind footpad. After four days, SRBC (10^8 cells) was injected into the left hind footpad. After 24 hours, the thickness of left hind footpad was measured by vernier calipers (Mitutoyo Co., 0.05 nm) (Fig. 6).

Effects of carrageenan on antitumor activity—Carrageenan (20 mg/kg, λ -form, Sigma) was injected intraperitoneally on the days -2 and 0, then sarcoma 180 cells (1×10^6 cell/mouse) were inoculated into the right flank of male ICR mice, 18-20 g, on the day 0 subcutaneously. Five mice were used for each group. Fraction A (40 mg/kg) was injected intraperitoneally everyday for five days, starting on the first day after the tumor implantation. To control group, saline was injected. After tumor implantation, tumor weights were measured on the 18th, 24th and 30th day.

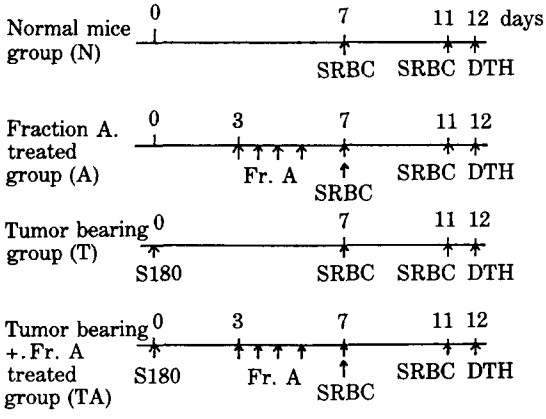

Fig. 6. Test schedule for effect of Fraction A on DTH response to SRBC.

RESULTS

Purification of antitumor components—The mycelia of the strain were fast grown in shake culture. When cultured in 15 *l* of the medium, a dark brownish power designated Fraction A (4.25g) was obtained. The elution profiles of the antitumor components were shown in Figs.7 and 8.

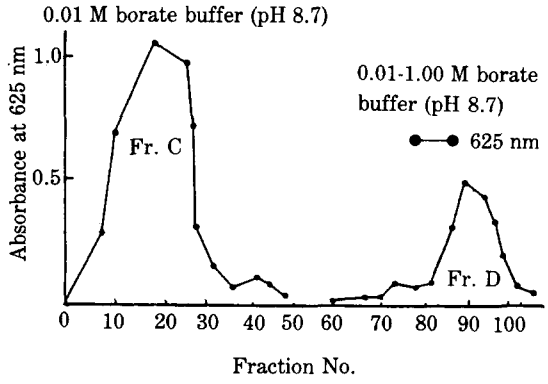

Fig. 7. DEAE-Sephadex A-50 (borate form) column chromatography of Fraction A obtained from *L. procera*.

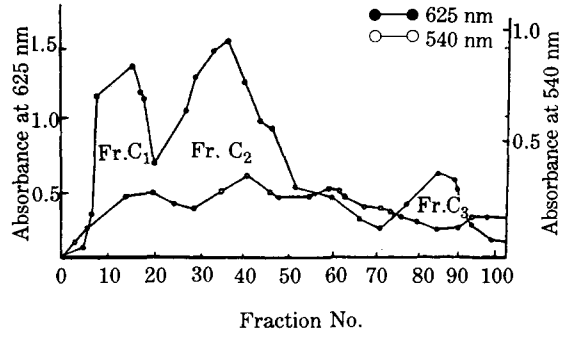

Fig. 8. Sepharose-4B gel filtration of Fraction C obtained from *L. procera*.

Table 1. Antitumor effects of the protein-polysaccharide fractions of *Lepiota procera*.

Sample	Dose (mg/kg/day, i.p.)	Tumor weight (g, mean ± S.D.)	Inhibition ratio (%)	Complete regression
Control	Saline	3.75 ± 0.46		
Fr. A	20×10	1.90 ± 1.34*	49.5	0/7**
Fr. E	20×10	3.42 ± 1.53	8.8	0/8
Control	Saline	4.36 ± 0.82		
Fr. C	20×10	2.49 ± 0.44	42.0	0/7
Fr. C_1	10×10	1.57 ± 0.17	64.0	1/7
Fr. C_2	20×10	2.12 ± 0.36	51.0	0/7
Control	Saline	3.60 ± 0.95		
Fr. B	10×10	1.57 ± 0.85	50.7	0/7

*$p < 0.001$ ** Number of mice used

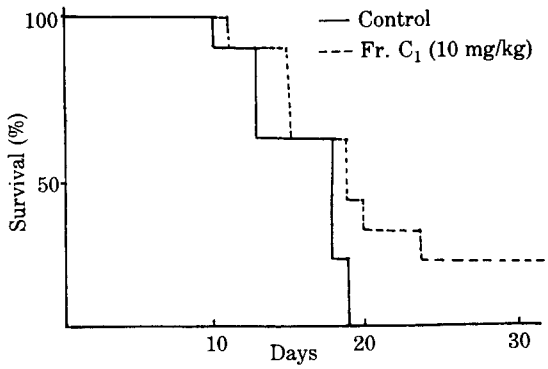

Fig. 9. Effects of the antitumor Fraction C_1 of *L. procera* on the life span after intraperitoneal implantation of sarcoma 180 in mice.

Table 2. Polysaccharide and protein contents of the antitumor components.

Fraction	Polysaccharide (%)	Protein (%)
Fr. A	17	10
Fr. B	78	8
Fr. C	53	23
Fr. C_1	60	21

Table 3. Monosaccharide contents of the polysaccharide moiety of the antitumor fractions

	Fr.A	Fr. C_1
Glucose	41.24	46.3
Fucose	18.3	11.0
Xylose	+	+
Mannose	39.2	40.2
Galactose	1.1	+

Table 4. Effects of the antitumor component on hemolytic plaque-forming cells in the spleen of ICR mice immunized with SRBC (1×10^7)

	Control	Treated
Body weight (g)	25.2 ± 1.1*	25.6 ± 0.3
Spleen weight (mg)	152.1 ± 1.5	178.0 ± 2.8
Spleen cell count (1×10^7)	8.5 ± 0.6	24.6 ± 0.3
PFC/10^6 spleen cells	4.7 ± 0.5	49.4 ± 1.4
PFC/spleen ($\times 10^2$)	4.0 ± 1.0	121.5 ± 22.5

* Mean ± standard deviation

Fraction A(2.1g) was separated into Fr. C(570 mg) and Fr. D(211 mg) by DEAE-Sephadex A-50 column chromatography. Fraction C (500 mg) was separated into Fr.C_1 (62 mg), Fr. C_2 (74 mg) by Sepharose-4B column chromatography.

Antitumor activity—Antitumor activity of the fractions obtained from *L. procera* on sarcoma 180 in mice was shown in Table 1. Of the six fractions tested, Fr. C_1 showed the highest inhibition ratio of 64.0% and complete regression was 1/7. Fig.9 showed the life span of the treated group and it was longer than that of control group. Upon administration of 10 mg/kg of Fr. C_1, 20% of the test mice survived for 30 days after tumor inoculation and the survival rate was 157%. Fr. C_1 was named lepiotan.

Chemical analysis—The contents of the total polysaccharide and total protein of the fractions were shown in Table 2. As shown in Table 3, the major monosaccharide subunits were glucose, fucose and mannose. Infrared spectra of the fractions were depicted in Fig. 10.

Effects on immune response–The PFC counts of the treated group showed about ten times higher

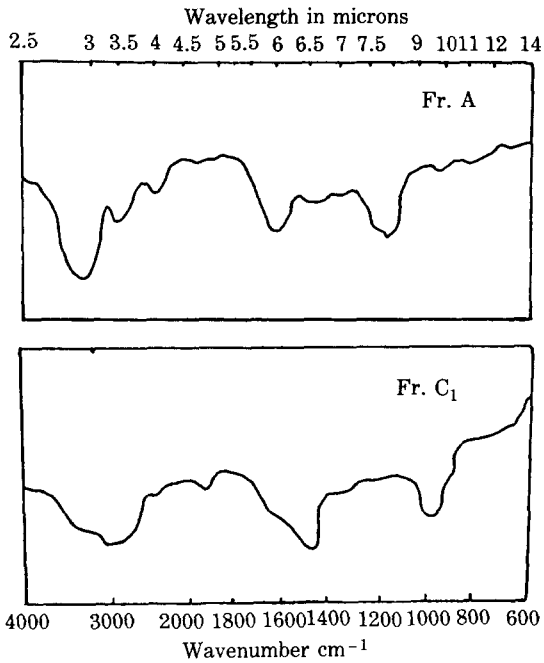

Fig. 10. I.R. spectra of the antitumor Fractions A and C_1 of *L. procera*.

Table 5. Effects of Fraction A on DTH response to SRBC.

Groups*	Footpad thickness (×0.1 mm±S.D.)	DTH response (%)
Normal mice group (N)	3.8±0.2	100
Fr. A treated group (A)	3.7±0.1	97.3
Tumor bearing group (T)	2.2±0.3	57.9
Tumor bearing + Fr. A treated group (TA)	3.4±0.1	89.4

* Each group included five mice.

than those of the control group and the results were summarized in Table 4.

And there was no difference between N group and A group in the effects on delayed type hypersensitivity. In T group, DTH was decreased 57.9% in comparision with N group and was restored 89.4% in TA group (Table 5).

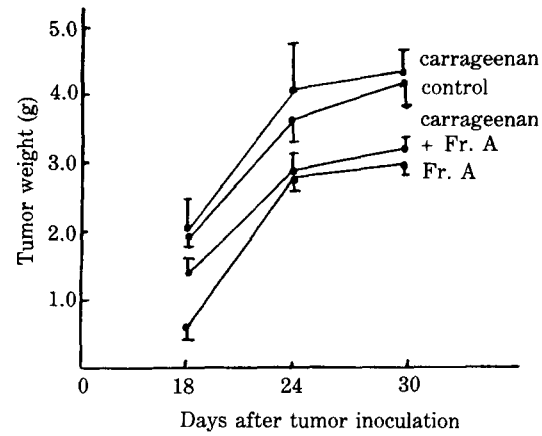

Fig. 11. Effect of carageenan on the antitumor activity of Fraction A of *L. procera*.
Each value was mean ± S.D.
Each group consisted of five mice.

And the tumor weights of the carrageenan treated group were higher than those of the control and the results were summarized in Fig. 11.

DISCUSSION

The protein—polysaccharides obtained from the cultured mycelia of *Lepiota procera* suppressed the growth of implanted sarcoma 180 when they were injected intraperitoneally into ICR mice. But the culture filtrate was ineffective. The purified antitumor component of Fr. C_1 showed inhibition ratio of 64% when administered at the dose of 10 mg/kg/day and was found more effective than the crude protein-polysaccharide. This fraction contained 60% polysaccharide and 21% protein. Although Fr. B was removed of its protein by Sevag method, it contained 8% protein. These results indicate that the protein and polysaccharide were bound fairly strongly.

In the IR spectra of Fr. A and Fr. C_1, O-H stretching frequency at 3300-3400 cm^{-1} and C-H streatching frequency at 2900 cm^{-1} and C-H, C-O bending frequency in 1000-1100 cm^{-1} and C-O stretching frequency at 1630 cm^{-1} were observed, and these characteristics were common to all these fractions. Although the structural features of the

extracted compounds were not elucidated, the characteristics were deduced from methylation, enzyme degradation and ^{13}C-NMR spectroscopy[21].

In order to elucidate mechanisms of the antitumor activity, the effects of this protein-polysaccharide on the immune response of mice immunized with SRBC were studied. The polymer was found to potentiate the production of hemolytic plaque-forming cells of the spleen. In addition, to examine its effects on cell-mediated immunity, delayed-type hypersensitivity (DTH) test was conducted. The polymer restored the suppressed delayed-type hypersensitivity in the tumor-bearing mice. Also the antitumor activity of Fraction A obtained from *L. procera* was reduced by treatment with carrageenan. Carrageenan, a high-molecular-weight sulfated polygalactose is toxic to macrophage and inhibits the function of macrophage *in vivo* and *in vitro*. Hence it is used as an anti-macrophage agent in immunological experiments. The results indicate that the antitumor activity is a macrophage-dependent response in the mice. Therefore its antitumor effect appears to be through host-mediated immune mechanism.

These results suggest that the antitumor action of this fraction can be regarded as an immuno-accelerating activity, but not as direct cytotoxic activity against sarcoma 180.

CONCLUSION

It was found that an antitumor constituent, named lepiotan, was isolated from the cultured mycelia of *Lepiota procera*. Lepiotan consisted of 60% polysaccharide and 21% protein. The polysaccharide moiety was composed of 46.3% glucose, 40.2% mannose and 11.0% fucose. It was shown that lepiotan exerted the antitumor activity through immunopotentiation.

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국문요약

한국산 담자균류인 갓버섯 *Lepiota procera*의 균사를 액내 배양하여 항암성분인 단백성 다당체를 분리하였다. 이 성분은 DEAE-Sephadex A-50 이온교환수지와 Sepharose-4B gel filtration을 이용하여 정제하여 Fraction C_1을 얻었으며 이 Fr. C_1은 단백질과 다당체로 구성되어 있으며 항암효과는 10 mg/kg/day 투여군에서 64%의 저지율을 나타내었다. 이러한 항암작용의 기전을 밝히기 위한 연구의 일환으로 면역에 미치는 영향을 실험한 결과 이 단백다당체는 용혈반형성 세포수를 증가시켰으며, 저하된 지연성 과민반응을 회복시켰을 뿐만 아니라, carrageenan 투여에 의해 억제된 면역능을 다시 증강시켰음을 알 수 있었다. 이러한 결과들은 이 버섯의 항암작용이 세포독성에 의한 것이 아니라 종양에 대한 면역능을 강화시켜 발휘됨을 제시하고 있다.

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