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Studies on Constituents of the Higher Fungi of Korea(XXXVIII)

Antitumor Components Extracted from Cultured Mycelia of *Pleurotus pulmonarius*

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韓國產 高等 菌類의 成分 硏究(第38報)

조개느타리버섯의 抗癌 成分

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Abstract : Antitumor components were obtained from the cultured mycelia of *Pleurotus pulmonarius* by ethanol precipitation. The protein-bound polysaccharide was subjected to DEAE-Sephadex column chromatography and Sephadex G-200 gel filtration. The antitumor fraction C_1 was isolated. The inhibition ratio of fraction C_1 was 81.8 % in the doses of 10 mg/kg/day for 10 days. The antitumor fraction C_1 consisted of a polysaccharide and a protein. The protein-moiety was composed of 14 amino acids. From the peritoneal cell populations in the mice given antitumor fraction C_1, the injection of the fraction caused the influx of peritoneal macrophages at two days when compared with those of soluble starch. This was named pulmonaran after its species name.

Keywords: *Pleurotus pulmonarius*, *Polyporaceae*, Basidiomycetes, Protein-bound polysaccharide, Pulmonaran, Antitumor components, Peritoneal macrophages, Shake culture of mycelia, Immune response.

Many antitumor preparations were obtained from a variety of natural sources such as higher plants (Nakahara *et al.*, 1964; Sakai *et al.*, 1964; Watanabe, 1966; Nakahara *et al.*, 1967; Kamasuka *et al.*, 1968), fungi (Chihara *et al.*, 1970), lichens (Nishikawa *et al.*, 1971), yeasts (Suzuki *et al.*, 1968) and bacteria (Shear, 1936). Karo (1930) reported the polysaccharides from some basidiomycetes in 1930. It was Lucas who first found the antitumor activity of the basidiomycetes in 1957 (Ringler *et al.*, 1957). Since then, active antitumor components such as lentinan, schizophyllan, flammulin and PS-K were reported. Although the mechanism of the antitumor activity of these components has not been completely elucidated, it was suggested that the action is not direct cytotoxicity on tumor cells (Maeda *et al.*, 1971) but through host-mediated immunity. Also there were many papers on the structure of antitumor polysaccharides and a branched (1−3)-β-D-glucan structure was responsible for the antitumor activity (Misaki *et al.*, 1981).

In recent years, our laboratory has been publishing the reports on the antitumor components obtained from the carpophores and cultured myc-

elia of Korean basidiomycetes (Kim *et al.*, 1976, 1978, 1979). *Pleurotus pulmonarius* is new to Korea and no report exists except that this fungus was one of the mushrooms which were grown on the potato juice concentrate as a substrate (Vecher *et al.*, 1979). 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, immunological effects of the purified antitumor component on the peritoneal cell populations in the mice were examined.

Materials and Methods

Shake Culture of Mycelia

1) Fungal Strain

The mycelia of *Pleurotus pulmonarius* (the family *Polyporaceae*) used in this experiment were kindly supplied from the Institute of Agricultural Sciences at Suwon in Gyeong-Gi Province.

2) Composition of Media

A) PDA Slant: Bacto potato dextrose agar (Difco Lab. U.S.A.) 39 g/L.

B) Shake Culture Medium: glucose 50 g, peptone 10 g, yeast ext. 10 g, KH_2PO_4 0.87 g, $MnSO_4 \cdot 7H_2O$ 0.5 g, $CaCl_2$ 0.3 g, $FeSO_4 \cdot 7H_2O$ 20 mg, $MnCl_2 \cdot 4H_2O$ 7 mg, $ZnSO_4 \cdot 7H_2O$ 4 mg, $CuSO_4 \cdot 5H_20$ 1 mg per L (pH 5.5).

3) Culture Methods

A) Seed Culture.

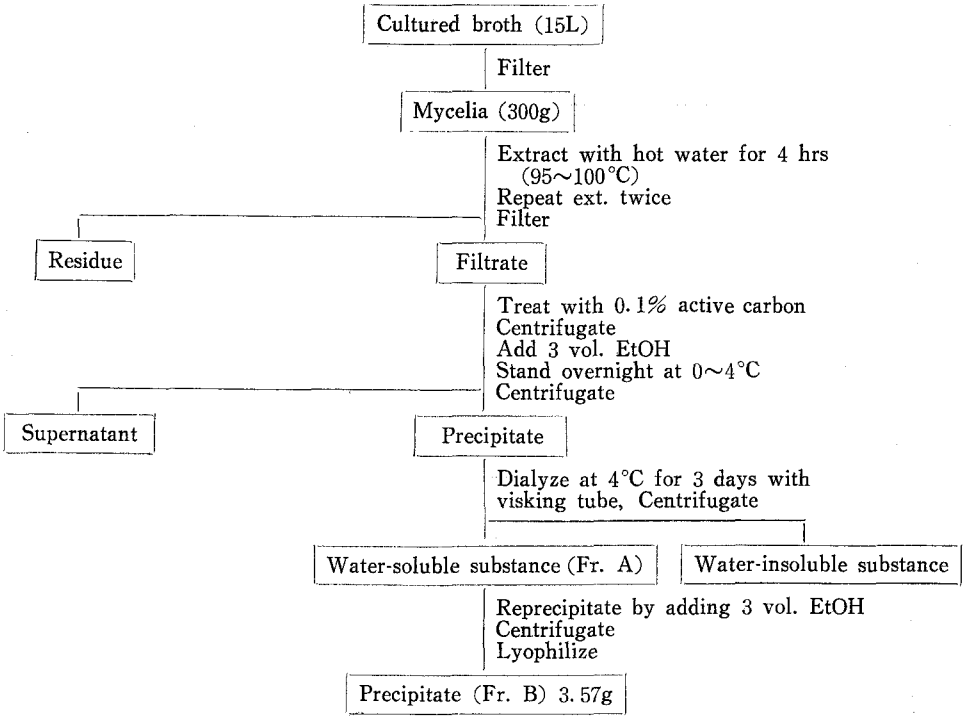
Mycelia of *P. pulmonarius* maintained on PDA slant were transferred aseptically on the new PDA slant and incubated for seven days at 26~28°C, *P. pulmonarius* grown on the new PDA slant was transferred into a microblender aseptically and homogenized for 10 seconds. One hundred and twenty five ml of the shake culture medium in a 500-ml flask was inoculated with 12.5 ml of the homogenized mycelia and incubated for seven days in an Orbital Shaking Incubator at 26~28°C, 180 rpm.

B) Main Culture

Culture broth including mycelia was homogenized, transferred aseptically to a 2-L flask containing 500 ml of the shake culture medium and shaken in the same method as previously described. The total thirty flasks were used for the shake culture.

Extraction of Antitumor Components

The mycelia from 15 L of the shake culture were collected on a Buchner funnel and washed three times with distilled water. Three hundred grams of the mycelia were homogenized and extracted two times with water on a water bath at 95~100°C for four hours. After filtration, filtrates were treated with 0.1 % active carbon and the active carbon was removed after one day. The filtrates were condensed in a rotary vacuum evaporator. Three volumes of 95 % ethanol were added to the concentrate and the mixtures allowed to stand at 4°C overnight. The precipitate was collected by centrifugation for 30 min. at 10,000 g and redissolved in water for dialysis. After dialysis at 4°C for three days using Visking tube, water-insoluble substances were removed by centrifugation and water-soluble substances were obtained. These water-soluble substances were designated as Fraction A. The water-soluble substances were reprecipitated by adding three volumes of 95 % ethanol and the precipitate was collected and lyophilized. The crude protein-bound polysaccharide was obtained as a brownish powder with a yield of 3.57 g. This brownish powder was designated as Fraction B (Scheme I).



Scheme I. Extraction of antitumor components from the cultured mycelia of *Pleurotus pulmonarius.*

Purification of Antitumor Components

1) DEAE-Sephadex A-50 Column Chromatography

A) Silanization of the Column

Dry column was rinsed twice with toluene and poured with 5 % dimethyldichlorosilane-toluene solution to the very top of the column to avoid resin adsorption on the column. The column was allowed to stand undisturbed for two hours in a hood. After emptying the column, this column was rinsed twice with toluene and then repeated with water.

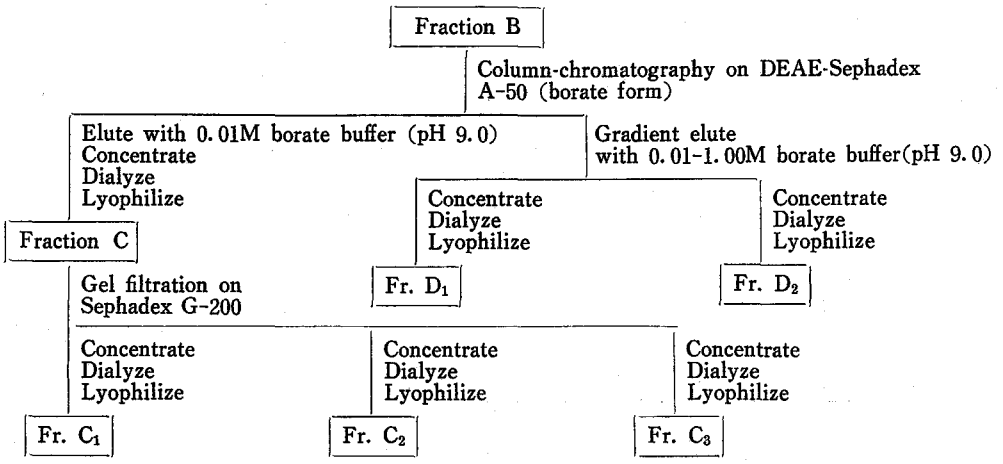
B) Preparation of the Ion Exchanger

DEAE-Sephadex A-50 resin (Pharmacia Fine Chemicals AB, Uppsala, Sweden, Cl^- form) was exchanged to borate form using 0.1 M boric acid. The fine particles were removed and DEAE-Sephadex A-50 (borate form) was swollen with fresh 0.01 M borate buffer (pH 9.0).

C) Column Chromatography

DEAE-Sephadex A-50 (borate form) was packed into a column (2.7×14 cm) with 0.01 M borate buffer (pH 9.0). A solution of Fraction B (3.3 g) in eluant (6 ml) was applied to the column. Then, it was eluted with 0.01 M borate buffer (pH 9.0) at a flow-rate of 20 $ml \cdot hr^{-1}$ and fractions of seven ml each were collected. Absorbance at 625 nm (Anthrone test) and 254 nm was measured. The Anthrone-positive fractions were concentrated, dialyzed and lyophilized. Thus, unadsorbate on DEAE-Sephadex was obtained as a white powder with a yield of 930 mg. This white powder was designated as Fraction C. Adsorbate on DEAE-Sephadex was eluted with 0.01-1.00 M linear gradient borate buffer (pH 9.0). The eluate was collected by the previous method. The yellow powders which were designated as Fraction D_1 (220 mg) and Fraction D_2 (250 mg) were obtained (Scheme II).

2) Sephadex G-200 Column Chromatography



Scheme II. Purification of antitumor components of *P. pulmonarius*.

Pre-swollen and degased Sephadex G-200 was packed into a column (2.5×65 cm) with 0.1 M tris-HCl buffer (pH 8.0) and 0.5 M NaCl. A solution of Fraction C (530 mg) in 0.01 M borate buffer (10 ml) was applied to the Sephadex gel column. Then, it was eluted with 0.1 M tris-HCl buffer (pH 8.0) containing 0.5 M NaCl at a flow-rate of 20 ml·h^{-1} and fractions of seven ml each were collected. Absorbance at 625 nm (Anthrone test) and 254 nm was measured. The Anthrone-positive fractions were concentrated, dialyzed and lyophilized.

The white powders which were designated as Fr. C_1, Fr. C_2 and Fr. C_3 were obtained (Scheme II).

Antitumor Test

1) Tumor Cells

Sarcoma 180 cells were implanted into the intraperitoneal cavity of male ICR mice weighing about 18~20 g. After one week, the animals were killed and sarcoma 180 ascitic fluid was collected. After washed three times with saline, ascitic tumor cells were adjusted to a concentration of 1×10^7 cells/ml.

2) Preparation of Test Solutions

Each eighty milligrams of Fr. A and Fr. B which were obtained from the cultured mycelia of *P. pulmonarius* were dissolved in 20 ml of saline for a dose of 20 mg/kg. Also, each forty milligrams of Fr. C, Fr. C_1 and Fr. C_3 were dissolved in 20 ml of saline for a dose of 10 mg/kg. Physiological saline was used for control.

3) Antitumor Test

Sarcoma 180 cells (1×10^6 cells/0.1ml/mouse) were implanted subcutaneously into the left groin of male ICR mice weighing about 18~20 g. Eight mice were used for each group.

The test solutions of Fr. A, Fr. B, Fr. C, Fr. C_1 and Fr. C_3 were respectively injected intraperitoneally every day 10 days at the doses of 20, 20, 10, 10 and 10 mg/kg/day, starting on the third day after the tumor implantation. To the control group, saline was injected (Scheme III). Tumor weights were measured on the 28th day after tumor implantation and tumor inhibition ratio was calculated as follows:

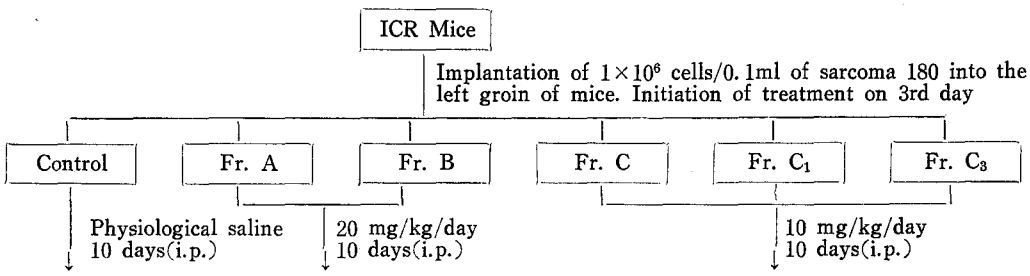
Tumor Inhibition Ratio

$$(\%)=\frac{C_w-T_w}{C_w}\times100$$

T_w=Average tumor weight of the treated group

C_w=Average tumor weight of the control group

4) Life Span



Scheme III. Antitumor test of the various fractions of *P. pulmonarius* on sarcoma 180.

Sarcoma 180 cells (5×10^5 cells/0.1ml/mouse) were implanted into the peritoneal cavity of male ICR mice weighing about 25 g. Eighteen 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 doses of 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 32 days.

Assay for Antitumor Components

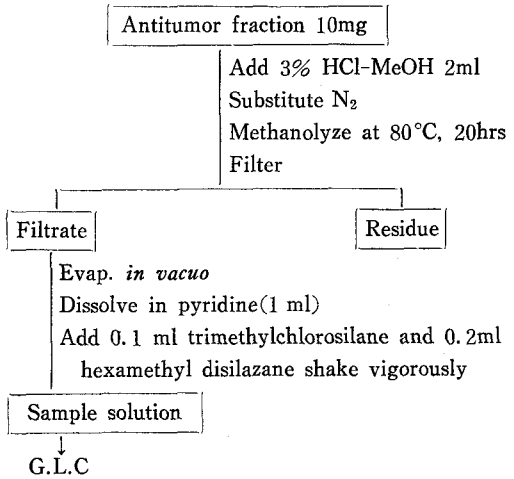
1) Analysis of Polysaccharide Components

A) Total Polysaccharide Content

The polysaccharide contents of the antitumor fractions were determined by the Anthrone method using glucose as a standard sugar. The absorbance at 625 nm was measured by U.V. Spectrophotometer. The polysaccharide content was calculated from the calibration curve.

B) Monosaccharide Analysis

To identify and quantify monosaccharide of the polysaccharide moiety, 10 mg of the antitumor fractions was dissolved in two ml of 3 % HCl-MeOH and methanolyzed at 80±5°C for 20 hours in a cap tube filled with nitrogen gas. The filtrate was evaporated in vacuum and dissolved in one ml of pyridine. For trimethylsilylation, hexamethyldisilazane (0.2 ml) and trimethylchlorosilane (0.1 ml) were added to pyridine solution and shaked vigorously for 30 seconds. And then, gas liquid chromatography was carried out. To identify the monosaccharide, retention times of the antitumor fractions were compared with those of standard monosaccharides. The content of each monosaccharide was calculated by weighing the peak area (Scheme IV).



Scheme IV. Sample preparation for G.L.C.

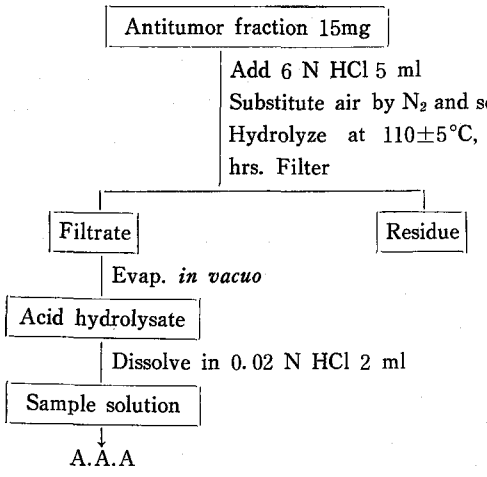
2) Analysis of Protein Components

A) Total Protein Content

The protein contents of antitumor fractions were determined by Lowry-Folin method using albumin as a standard protein. The absorbance at 725 nm was measured by U.V. Spectrophotometer. The protein content was calculated from the calibration curve.

B) Amino Acid Analysis

To determine the identity and content of the



Scheme V. Sample preparation for amino acid analysis.

amino acids, 15 mg of the antitumor fractions were dissolved in five ml of 6 N-hydrochloric acid in an ampule. After nitrogen gas substitution and sealing of the ampule, the sample was hydrolyzed at 110±5°C for 24 hours. After filtration, the filtrate was evaporated *in vacuo* and dissolved in two ml of 0.02 N-hydrochloric acid. This sample solution (0.5 ml) was injected to Hitachi KLA-5 amino acid analyzer. Amino acids were detected and quantified by comparison with the chromatogram of the standard amino acids. The content of each amino acid was calculated by half width method (Scheme V).

Test of Antitumor Components on Immune Response

1) Animals

ICR mice weighing 25~28 g of male were supplied from the Experimental Animal Farm of Seoul National University.

2) Materials

Phosphate-buffered (0.01 M) saline (PBS), pH 7.2~7.4

Balanced salt solution (BSS)

Stock I: Dextrose 10 g, KH_2PO_4 0.6 g, Na_2HPO_4 anhyd. 1.85 g, 0.5 % phenol red soln 2 ml per L.

Stock II: $CaCl_2$ anhyd. 1.86 g, KCl 4.0 g, NaCl 80 g, $MgCl_2$ anhyd. 2.0 g, $MgSO_4$ anhyd. 2.0 g per L.

When using, added 800 ml distilled water to mixtures of 100 ml stock I and 100 ml stock II and adjusted to pH 7.2

Incubation mixtures

Stock solution: Dissolved 1 g of α-naphthyl acetate in 50 ml distilled water and added 50 ml acetone.

Working solution: Added two ml of stock solution and 15 ml of 0.1 M phosphate buffer (pH 7.3) and 20 mg of fast red TR salt in 15 ml distilled water.

Before use, mixtures were filtered and used immediately.

Giemsa solution

Stock Giemsa: To 33 ml absolute methanol and 33 ml glycerol, added 0.5 g Giemsa powder. Mixed with a magnetic stirrer for 15 min, four times daily for three days. Filtered and storeed in brown bottle at room temperature.

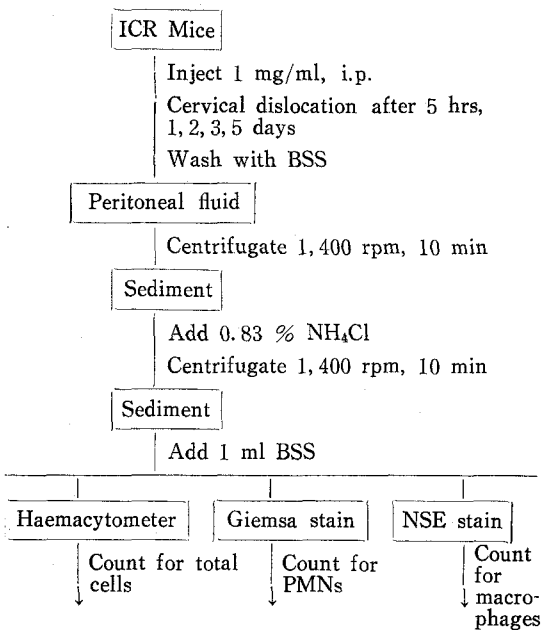
Working Giemsa: To 4 ml stock Giemsa stain, added 60 ml distilled water and adjusted to pH 7.0~7.2.

0.83 % Aqueous ammonium chloride: used for erythrocytes lysis.

1 % Malachite green solution: used for counter-staining in NSE stain.

3) Procedure

Thirty mice were divided into five groups. Three mice of one group were injected intraperitoneally with one ml antitumor fraction C_1. Injection volume was one ml/mouse and control group received soluble starch. It was injected in the same method as previously described. After five hrs, one day, two days, three days and five days, mice were sacrificed by cervical dislocation. Peritoneum was washed with BSS and the washed fluid from peritoneum was collected into centrifuge tubes on ice. The tubes were centrifugated



Scheme VI. Procedure for comparison of peritoneal cell populations given antitumor fraction C_1.

at 240×g for 10 min, and the supernatant was decanted and the erythrocytes were lyzed with 0.83 % aqueous ammonium chloride. The sediment was resuspended gently with one ml of BSS for counting total cells, macrophages and polymorphonuclear leucocytes (PMNs). The total cells were counted in the haemacytometer. The cells which bear reddish granules after NSE stain were counted as macrophages and cells with multi-lobbed nuclei were counted as PMNs (Scheme VI).

Results

Shake Culture of Mycelia

The mycelia of *Pleurotus pulmonarius* was fast grown in shake culture media and its yield was 300 g when cultured to 15 L.

Purification of Antitumor Components

The elution profile of antitumor components was shown in Figs. 1 and 2. Fraction B (3.3 g) was separated into Fr. C (930 mg), Fr. D_1 (22 mg) and Fr. D_2 (250 mg) by DEAE-Sephadex A-50 column chromatography. Fraction C (530 mg) was separated into Fr. C_1 (350 mg), Fr. C_2 (21 mg) and Fr. C_3 (43 mg) by Sephadex G-200 column chromatography.

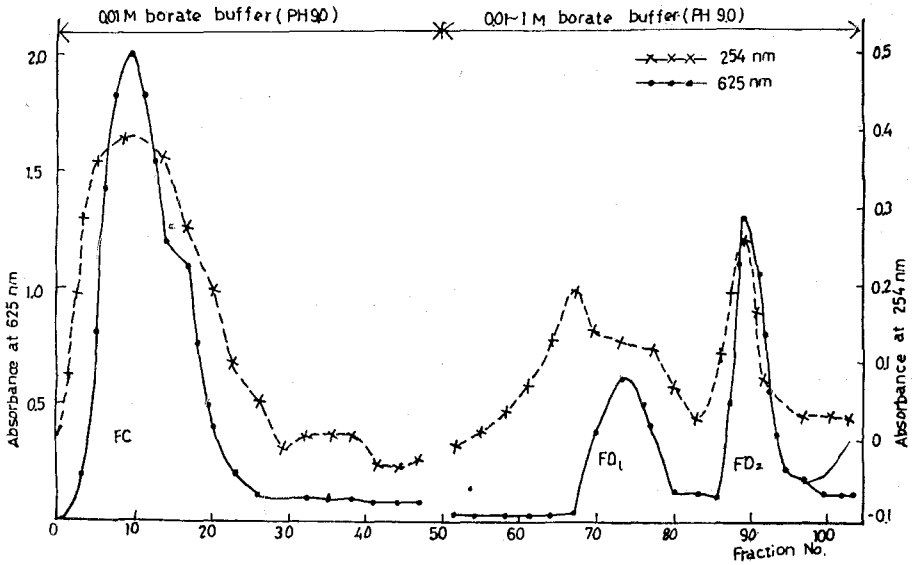


Fig. 1. DEAE-Sephadex A-50 (borate form) column chromatography of Fraction B obtained from *P. pulmonarius*.

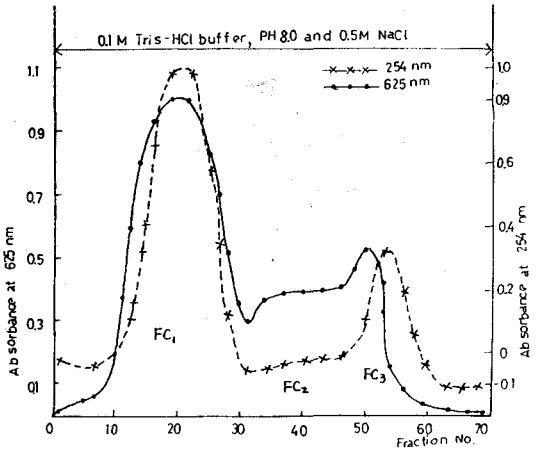


Fig. 2. Sephadex G-200 gel filtration of Fraction C obtained from *P. pulmonarius*.

Antitumor Activity

Antitumor activity of the fractions obtained from *P. pulmonarius* on sarcoma 180 in mice was shown in Table I. The inhibition ratios of Fr. A and Fr. B, which were found to be crude protein-bound polysaccharides, were respectively 52.6 % and 51.0 %. The inhibition ratios of Fr. C, which was the unadsorbate on DEAE-sepha dex, was 81.8 % and those of Fr. C_1 and Fr. C_3 which were purified by sephadex G-200 were respectively 81.1 % and 19.0 %. Fig. 3 depicted the life-span prolongation effect of the antitumor fraction C_1 on sarcoma 180-bearing mice. The mortality of mice given Fraction C_1 in comparison with saline was also reduced.

Chemical Composition of the Antitumor Components

1) Contents of Polysaccharide

Table II shows the total polysaccharide content determined by Anthrone test and the content of its monosacharides. Fig. 4 shows the G.L.C. pattern of the monosaccharides. Glucose was identified as a major subunit of the polysaccharide moiety of the fractions.

2) Contents of Protein

Table III shows the total protein content by Lowry-Folin method and the contents of its amino acids. The major amino acids were aspartic

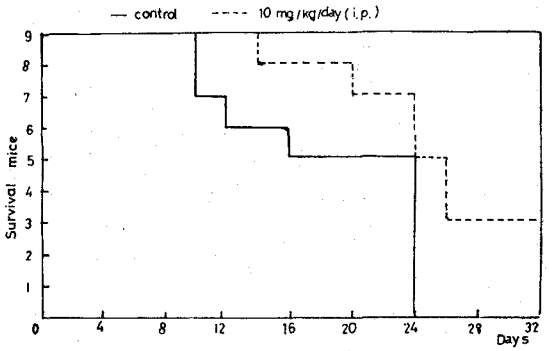


Fig. 3. Effects of the antitumor fraction C_1 of *P. pulmonarius* on the life span after intraperitoneal implantation of sarcoma 180 in mice.

Table I. Antitumor effects of the protein-bound polysaccharide fractions from the cultured mycelia of *P. pulmonarius* on sarcoma 180 in mice.

Sample	Dose (mg/kg/day, i.p.)	Average tumor weight (g)	Inhibition ratio (%)	Complete regression
Control	saline	2.85±0.55*	—	0/8**
Fraction A	20	1.35±0.26	52.6	0/7
Control	saline	2.25±0.28	—	0/8
Fraction B	20	1.10±0.47	51.0	1/8
Fraction C	10	0.41±0.13	81.8	2/8
Control	saline	4.03±1.73	—	0/8
Fraction C_1	10	0.73±0.28	81.8	0/8
Fraction C_3	10	3.24±1.35	19.0	0/8

* Values are means ± standard error. ** Number of mice used

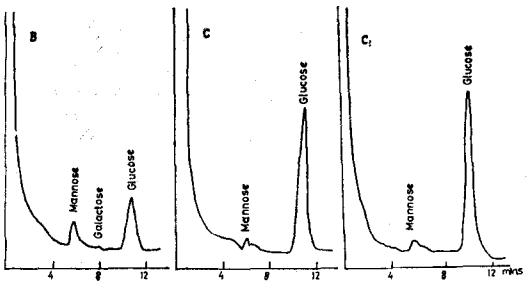


Fig. 4. G.L.C pattern of the monosoccharides in the antitumor fractions B, C and C_1 of *P. pulmonarius*.

Table II. Polysaccharide and monosaccharide contents of the antitumor fractions of *P. pulmonarius*.

Fraction	B	C	C_1
Polysaccharide content(%)	67	80	82
Monosaccharide content(%)			
Glucose	70	90	91
Mannose	24	10	9
Galactose	6	0	0

Table III. Protein and amino acid contents of the antitumor fractions of *P. pulmonarius*.

Fraction	B	C	C_1
Protein content(%)	5	2	1
Amino acid content(%)			
Histidine	15.0	8.8	—
Serine	14.3	8.8	6.8
Alanine	8.8	3.4	4.0
Glutamic acid	7.8	9.0	10.5
Lysine	7.6	4.5	29.8
Phenylalanine	6.8	16.2	15.0
Threonine	6.7	7.9	6.4
Aspartic acid	6.4	6.5	7.2
Tryptophan	5.2	2.2	—
Proline	4.9	1.7	0.3
Valine	4.5	17.2	5.3
Glycine	2.9	0.9	1.4
Isoleucine	2.8	2.4	1.8
Leucine	2.5	2.2	3.0
Arginine	2.2	6.0	3.9
Methionine	1.0	—	—
Tyrosine	0.6	2.3	4.6

* Ammonia was also detected.

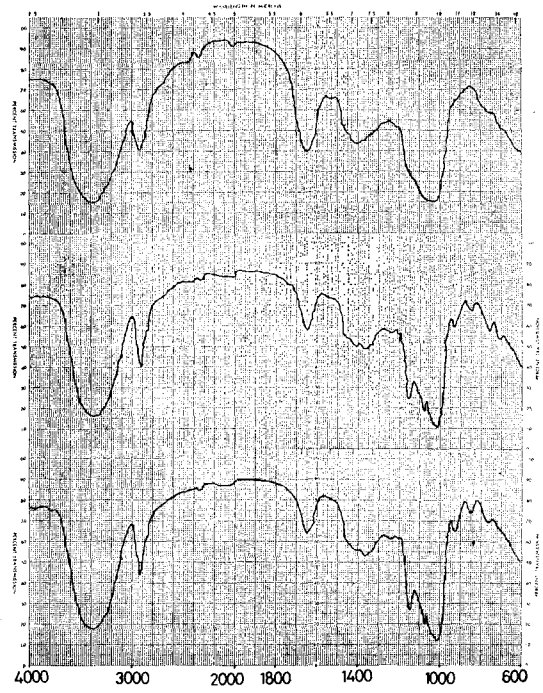


Fig. 5. Infrared spectra of the antitumor Fractions B, C and C_1 of *P. pulmonarius*.

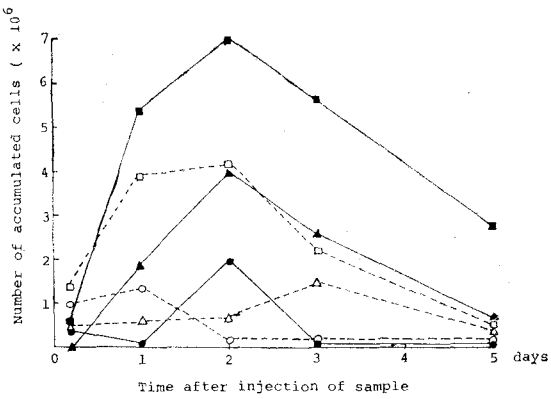


Fig. 6. Kinetics and composition of peritoneal exudate cells after injection of the antitumor Fraction C_1 of *P. pulmonarius*.
■ (□) Total Cells, ▲ (△) Macrophages, ● (○) PMNs, ■ ▲ ● Antitumor Fraction C_1, □ △ ○ Soluble Starch

acid, threonine, serine, glutamic acid and phenylalanine.

3) Infrared Spectra

Fig. 5 depicts the infrared spectra of Fr. B (crude protein-bound polysaccharide), Fr. C (purified by DEAE-Sephadex) and Fr. C_1 (purified by Sephadex G-200).

Effects of the Antitumor Fraction on Peritoneal Cell Populations

The effect of Fraction C_1 on the macrophage accumulation by comparison with soluble starch was shown in Fig. 6. The counts of peritoneal exudate cells and macrophages reached the maximum levels after two days and gradually decreased. PMNs also reached the maximum level after two days and reached the level of normal mice after three days.

Discussion

The preparations obtained from the cultured mycelia of *Pleurotus pulmonarius* suppressed the growth of implanted sarcoma 180 when they were injected intraperitoneally into ICR mice.

The purified antitumor components of Fractions C and C_1 exhibited equal inhibition ratio of 81.8 % when administered at the dose of 10 mg/kg/day and were found more effective than the crude protein-polysaccharide fraction.

As shown in Tables Ⅱ and Ⅲ, the monosaccharides and amino acids of Fractions C and C_1 were almost equal and infrared spectra of these fractions were very similar and their chemical composition resembled that of *Ganoderma lucidum*. The inhibition ratio of *Ganoderma lucidum* was 87.6 % at the dose of 50 mg/kg/day.

In the IR spectra of Fractions B, C and C_1, O-H stretching frequency at 3,300~3,400 cm^{-1} and C-H stretching frequency at 2,900 cm^{-1} and C-H, C-O bending frequency in 1,000~1,100 cm^{-1} and C-O stretching frequency at 1,630 cm^{-1} were observed and these characteristics were common to all these fractions.

In addition, peritoneal exudate cells and macrophages of the mice given Fraction C_1 greatly increased as compared to those of the mice given soluble starch.

This fact suggests that the antitumor mechanism of Fraction C_1 obtained from *P. pulmonarius* may be through the immunological potentiation involving macrophages. Although the increase of the macrophages was observed in the mice, it was not clearly clarified in this experiment whether these macrophages attack the sarcoma cells, since the sarcoma cells had not been implanted simultaneously into the mice in which the macrophage increase was examined.

More detailed immunological studies on a possible relationship between the accumulated macrophages and lymphocytes, and on immunomodulating activity of the T-and B-lymphocytes would be needed to elucidate clearly antitumor mechanism of Fraction C_1 of *P. pulmonarius*.

Conclusions

The protein-bound polysaccharide obtained from the cultured mycelia of *Pleurotus pulmonarius* suppressed the growth of sarcoma 180 in mice. The inhibition ratio of the crude protein-bound polysaccharide was 51.0 % at the dose of 20 mg/kg/day and that of the highly purified fraction C_1 was 81.8 % at the dose of 10 mg/kg/day. The highly purfied fraction C_1 consisted of a polysaccharide and a protein and the polysaccharide was composed of two monosaccharides and the protein contained 14 amino acids. The antitumor fraction C_1 increased the macrophages in the peritoneal cavity of the mice than soluble starch. This fact suggests that the fraction C_1 may be an immunopotentiator. This was named pulmonaran after its species name.

Acknowledgments

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要 約

조개느타리 *Pleurotus pulmonarius*을 액내 배양하여 항암 성분인 단백성 다당체를 얻었다. 이 성분의 항암효과는 20 mg/kg/day 투여군에서 51.0 %의 저지율을 나타내었다. 또한 단백성 다당체는 DEAE-Sephadex 이온 교환수지와 Sephadex G-200을 이용하여 정제하였고 여기서 Fraction C_1인 항암성분을 얻었다. 이 항암성분은 다당체와 단백질로 구성되어 있었으며 항암효과는 10 mg/kg/day 투여군에서 81.8 %의 저지율을 보였다. 이런 항암작용의 기전을 알기 위해 복강내에 Fraction C_1을 투여하고 이에 대한 복강세포의 증가를 관찰한 결과 면역세포 중 macrophage의 증가가 두드러졌으며 이는 soluble starch와 비교해 볼 때 훨씬 많은 macrophage 증가 효과가 있음을 증명하였다. 이 성분을 pulmonaran이라 명명하였다.

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