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Studies on Constituents of the Higher Fungi of Korea (LI) Antitumor Components Extracted from the Cultured Mycelia of *Volvariella bombycina*

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=국문초록=

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흰비단털버섯 *Volvariella bombycina*의 균사체를 대량으로 액내 배양 진탕한 후 얻은 균사체를 증류수로 추출하고, ethanol로 침전시켜 분리, 정제한 고분자 성분이, sarcoma 180 세포를 피하에 이식한 ICR 마우스에서 60.3%라는 종양 저지율을 나타내었다. Sepharose 4B에서 gel filtration을 시행한 후 얻은 각 분획들을 항암 실험을 시행한 결과, Fraction B가 가장 높은 저지율을 나타냈다. Fraction B를 화학 분석한 결과, 이것은 단백질과 다당체로 구성된 단백 결합 다당체이었다. 또한 항암 작용 기전을 밝히기 위한 연구의 일환으로 면역에 미치는 영향을 실험한 결과, Fraction A는 복강내에서 macrophage의 수를 증가시켰을 뿐 아니라, 용혈반 형성 세포의 수도 현저히 증가시켰다.

INTRODUCTION

The antitumor activities of polysaccharide preparations from various natural sources, such as higher plants[1~3], fungi[4~5], lichens[6], and bacteria, have been reported in addition to the well-known yeast cell wall polysaccharide, zymosan.[7]

The antitumor activity of Basidiomycetes was first demonstrated by Gregory and his collaborators, who employed extracts of fruiting bodies of the mushroom *Boletus edulis* (Bull.) Fr.[8] Various kinds of Basidiomycete preparations which include: lentinan[9], a high molecular weight β-1, 3 glucan obtained from *Lentinus edodes* fruit bodies; schizophyllan[10], a high molecular weight β-1, 3: 1, 6 glucan prepared from *Schizophyllum commune* culture filtrates; and PSK[11], a peptide containing β-1, 4 : 1, 3 or β-1, 4 : 1, 6 glucan extracted from *Coriolus versicolor* culture mycelia, were shown to exhibit antitumor activity. These compounds are considered to exert antitumor activity through potentiation of the host animal's defense mechanism rather than direct inhibition of the tumor growth[12].

In recent years, studies on the antitumor

activities of several protein-polysaccharides from the carpophores and cultured mycelia of Korean Basidiomycetes have been already carried out in our laboratory[13~16].

In the present experiments, the hot water extract obtained from the cultured mycelia of *Volvariella bombycina* showed potent antitumor activity against sarcoma 180 in ICR mice. It was further purified by Sepharose 4B column chromatography based on the antitumor activity.

In addition, to study the mode of the action, its effects on immune responses were examined. In the course of these studies, it was noted that the protein-polysaccharide fraction affected the reactivity of peritoneal exudate cells(PEC) and antibody production.

MATERIALS AND METHODS

1) Materials

The strain of *Volvariella bombycina* (the family *Volvariaceae*) used in this work was provided by Agricultural Science Institute at Suweon, Gyeong-Gi Province.

2) Medium Composition

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

(2) Culture Medium: Glucose 50 g, peptone 10 g, yeast ext. 10 g, KH_2PO_4 0.87 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCl_2$ 0.3 g, $FeSO_4 \cdot 7H_2O$ 10 mg, $MnCl_2 \cdot 4H_2O$ 7 mg, $ZnSO_4 \cdot 7H_2O$ 4 mg, $CuSO_4 \cdot 5H_2O$ 1 mg per liter. It was adjusted to pH 5.5 and autoclaved at 121°C and 2 atm for 15 min.

3) Culture Methods

(1) First Culture: The mycelia of *V. bombycina* were aseptically transferred into a fresh PDA slant and was cultured for seven days at 27±1°C. The 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 100 ml of the culture medium in a 500-ml flask and incubated for 10 days in an orbital shaking incubator at 27±1°C and 180 rpm.

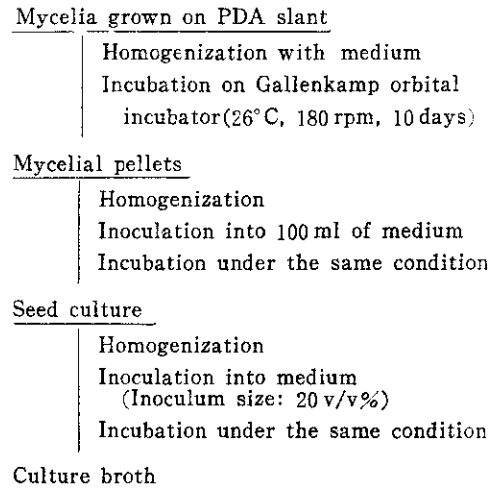
(2) Second Culture: The obtained culture pellets were aseptically homogenized for 10 seconds and inoculated into a 500-ml flask containing 100 ml of the culture medium.

Incubation was carried out under the same condition of the first culture for 10 days.

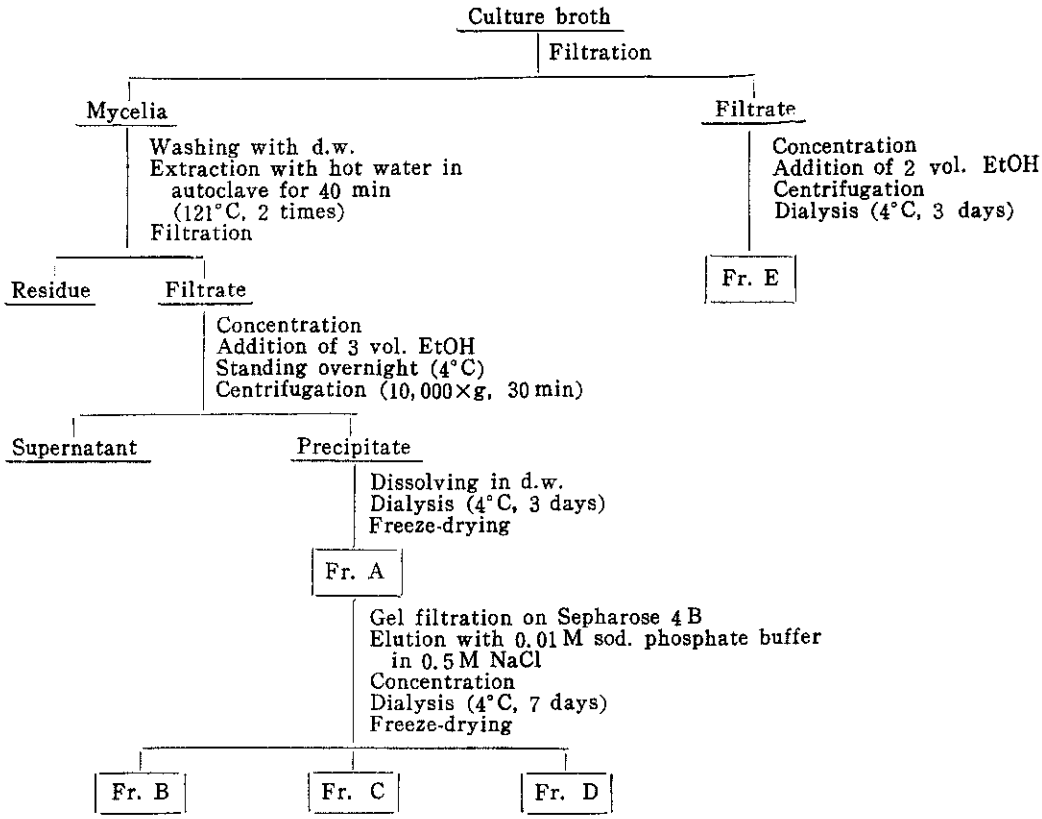
(3) Main Culture: Again, the obtained mycelial pellets from the second culture were aseptically homogenized for 10 seconds and transferred into 500 ml of the culture medium in a two liter flask (inoculum size: 20 v/v%) and cultured for 10 days in the same condition as previously described (Scheme I).

4) Extraction and Separation

Mycelia obtained from 15 L of the culture broth were filtered and washed twice with



Scheme I. Culture method of *Volvariella bombycina*



Scheme Ⅱ. Isolation and purification of the polysaccharide fraction from the metabolites of *V. bombycina*

distilled water (=d.w.). The mycelia were homogenized and extracted with d.w. in an autoclave (121°C, 2 atms) for 30 min. This process was repeated. The combined extracts were precipitated by adding three vol. of ethanol and allowed to stand at 4°C overnight. The precipitates were collected by centrifugation and redissolved in d.w. and dialyzed at 4°C for three days using visking tube (36/32). The precipitates formed during the dialysis were removed by filtration. The filtrates were concentrated and lyophilized to obtain a brownish powder with a yield of 2.7 g. It was designated as Fraction A.

The filtrates obtained from the culture broth were also treated as the above method except two vol. of 95% ethanol. It was designated as Fraction E (0.67 g/L).

5) Purification of Antitumor Components

Of Fraction A, 1.2 g was dissolved in 20 ml of 0.01 M sodium phosphate buffer (pH 7.3) and applied to a column (2×45 cm) of Sepharose 4 B (Sigma). The column was eluted with 0.01 M sod. phosphate buffer containing 0.5 M NaCl at a flow rate of 5 ml per 30 min[16~17].

Each effluent was analyzed for total sugar by anthrone method at 625 nm. The aliquots of each fraction were combined, dialyzed and lyophilized (Scheme Ⅱ).

6) Antitumor Test

(1) Animal: Male ICR mice (18~20 g) were supplied from the Experimental Animal Farm

ICR mouse with sarcoma 180 (ascitic form)
- Sacrificing with $CHCl_3$ vapor
- Collecting sarcoma 180 cells with ice cold saline
- Centrifugation (400×g, 5 min)

Cytocentrifugate
- Washing with ice cold saline (×3)
- Dilution to 1×10^7 cell/ml
- Inoculation with 0.1 ml of sarcoma 180 cell into right-flank (*s.c.*)

ICR mice implanted with sarcoma 180 cells
- After four days, sample injection (20 mg/kg, *i.p.*, once daily for consecutive 10 days)
- Sacrificing 26 days after the tumor implantation
- Excising the tumors

Solid tumors

Scheme Ⅲ. Antitumor test procedure *in vivo*

of Seoul National University.

(2) Tumor: Sarcoma 180 was used for testing the antitumor activity of *V. bombycina in vivo*. It was maintained continuously in our laboratory.

(3) Transplantation of Tumor Cells: Tumor cells (1×10^6)/mouse were inoculated subcutaneously into the right-flank of mice. Each fraction was administered intraperitoneally from the fourth day after tumor inoculation and once daily for 10 consecutive days(20 mg/kg) (Scheme Ⅲ).

(4) Evaluation of Inhibition Ratio: On the 26 th day after tumor inoculation, the mice were sacrificed and the solid tumors were dissected and weighed. The inhibition ratio was calculated as follows:

$$\text{Tumor inhibition ratio}(\%) = \frac{Cw-Tw}{Cw}\times100$$

—Cw: Average tumor weight of control group

—Tw: Average tumor weight of treated group

7) Effects of Fr. A on Immune Responses

(1) Effects on Peritoneal Cell Population:

① Animal: Male ICR mice(18~20 g) were provided by the Experimental Animal Farm of Seoul National University.

② Reagents:

(a) PBS(0.01 M Phosphate buffer saline) pH 7.2~7.4.

(b) BSS(Balanced salt solution)

Stock sol'n Ⅰ
- dextrose 10 g, KH_2PO_4 0.6 g
- $Na_2HPO_4\cdot7H_2O$ 3.58 g
- 0.5% phenol red solution 20 ml/L

Stock sol'n Ⅱ
- $CaCl_2$ anhyd. 1.86 g, KCl 4.0 g
- NaCl 80 g, $MgCl_2\cdot$anhyd. 2.0 g/L

BSS working sol'n
- stock sol'n Ⅰ 100 ml
- stock sol'n Ⅱ 100 ml
- demineralized water 800 ml
- mixed sol'n was adjusted to pH 7.3

(c) NSE staining solution(Non-specific esterase)

Stock sol'n
- α-naphthyl acetate 1 g
- acetone 50 ml
- distilled water 50 ml

Working sol'n
- stock sol'n 2 ml
- fast red TR salt 20 mg
- 0.1 M phosphate buffer (pH 7.3) 15 ml
- distilled water 15 ml

Before use, the mixtures were filtered and used immediately.

(d) Giemsa staining solution

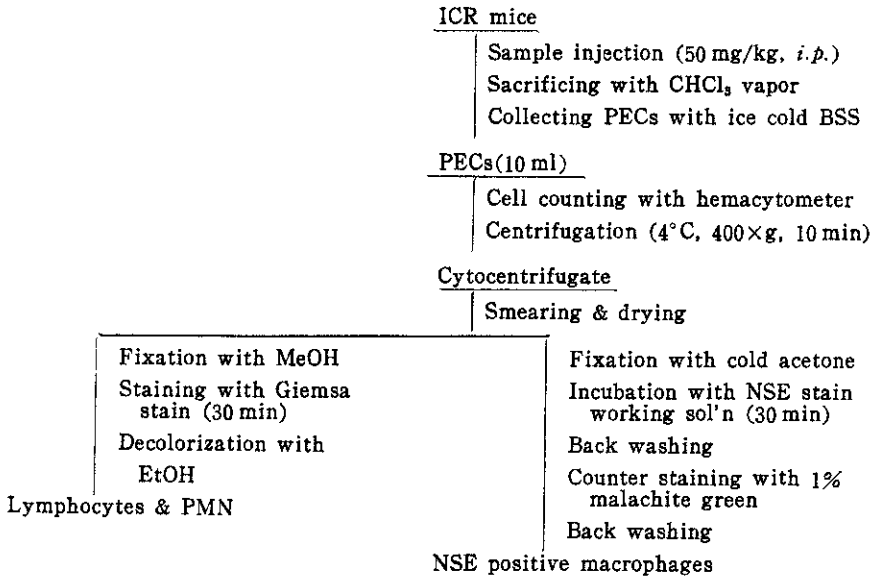
③ Methods:

(a) Sample administration

Fraction A was diluted in PBS and was injected intraperitoneally at a dose of 1 mg/ml(50 mg/kg).

(b) Preparation of peritoneal exudate cells

After one day, two days, three days and



Scheme Ⅳ. Procedure for macrophage accumulation test

five days of sample administration, peritoneal exudate cells were obtained by washing the peritoneal cavity with BSS(100 ml/total volume). The numbers of PEC were counted directly by a hemacytometer.

(c) Lymphocyte and PMN count

After PEC count, the peritoneal fluid was centrifugated at 4°C, 400×g for 10 min and the cytocentrifugates were suspended into 0.2 ml of ice-cold BSS. The PEC suspension was smeared on the slide glass, air-dried, fixed in MeOH for 5 min and stained with Giemsa sol'n for 30 min. The slides, to remove the dye, were rinsed in absolute ethanol and examined using Cedar oil as a mounting sol'n. The cells which showed blue color and round shape were counted as lymphocytes and the cells which contained a multi-lobbed and/or doughnut-type nucleus were counted as PMNs (polymorphonuclear leukocytes)[18].

(d) Macrophage count

The specimen on the glass slides were air-dried, fixed in cold acetone for five min and incubated with NSE working sol'n at 37°C for 30 min. The slides were back-washed with tap water and counter-stained with 1% malachite green staining sol'n for 15 seconds and back-washed with tap water again.

The slides were examined using glycerol jelly as a mounting solution. The cells which contained reddish granules were identified as non-specific esterase positive macrophage(Scheme Ⅳ).

(2) Effects on Hemolytic Plaque-Forming Cells:

① Animal: Male ICR mice(18~20 g) were supplied from the Experimental Animal Farm of Seoul National University.

② Materials:

(a) BSS

(b) 0.83% NH_4Cl solution

(c) Microchamber

After cleaning glass slides with 95% ethanol, two strips of double sided scotch tape were placed across both ends of the slides.

(d) SRBC (=sheep red blood cells)

SRBC was kindly supplied from Korea National Institute of Health.

(e) Complement

Guinea pig serum as a source of complement was also provided by Korea National Institute of Health.

③ Methods:

(a) Sample administration and immunization

Ten mice were divided into two groups. For a treated group, 50 mg of Fraction A was dissolved in five ml of saline and 0.1 ml of this solution was injected intraperitoneally once a day for five consecutive days.

On the seventh day after the last sample administration, the mice were immunized by intraperitoneal injection of 1×10^7 of SRBC.

(b) Preparation of spleen cell suspension

Five days later, the mice were sacrificed, and the spleens were dissected. The spleens were homogenized with ice-cold BSS and centrifugated at 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 resuspended in ice-cold BSS. The spleen cells were counted directly by hemacytometer.

(c) Preparation of complement-SRBC

SRBC was centrifugated and resuspended in saline to adjust the concentration into 20 v/v%. And then, 500 mcl of 20 v/v% SRBC were mixed with 1,000 mcl of guinea pig serum in the microwell and the fixation was carried out on the ice bath.

(d) Preparation of incubation mixture

Incubation mixture consisted of 150 mcl of complement-SRBC solution and 640 mcl of spleen cell suspension and 100 mcl of this mixture were placed into the microchamber (Scheme V)[19~21].

(e) Incubation and reading of results

ICR mice
- Sample injection (50 mg/kg, 5 days, *i.p.*)
- After 7 days, immunization by injecting 1×10^7 SRBC
- After 5 days, excising of spleen

Spleen
- Homogenization with ice cold BSS
- Centrifugation (400×g, 5 min)

Spleen cell
- Hemolysis with 0.83% NH_4Cl sol'n
- Centrifugation (400×g, 5 min)

Spleen cell without erythrocytes
- Washing with BSS (×4)
- Dilution with BSS

Spleen cell suspension
- Addition of 150 mcl of prepared suspension SRBC (100 mcl) + complement from guinea pig serum (50 mcl)
- Mixing
- Filling the microchamber
- Sealing
- Incubation at 37°C for one hour

Hemolytic plaque forming cells

Scheme V. Procedure of hemolytic plaque assay

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:

$$\text{PFC}/10^5 \text{ spleen cells} = \frac{N}{C\cdot Vm\cdot a}\times10^6$$

PFC/total spleen cells
= PFC/10^6 spleen cells × C × Vs

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

N: number of plaques observed in one microchamber

C: count of spleen cells in 1 ml of spleen cell suspension

Vm: volume of incubation mixture filled into one microchamber (ml)

Vs: total volume of spleen cell suspension (ml)

Table 1. Running conditions of G.L.C.

Column	3% OV-1 (80~100 mesh shimalite)	
	Boronsilicate glass column (2 mmϕ×1.0 m)	
Temperature	Column (isothermal)	160°C
	Injector	290°C
Flow rate	N_2: 50 ml/min	
	H_2: 0.5 kg/cm^2	
	Air: 0.6 kg/cm^2	
Range	10^2	
Attenuate	2^2	
Model	Shimadzu G.C. RIA	

8) Chemical Analysis

(1) Total Polysaccharide Content: Total polysaccharide contents were quantitatively determined by anthrone test using D-glucose as a standard. After anthrone test, the polysaccharide content was calculated from U.V. absorbance at 625 nm[22].

(2) Total Protein Content: Total protein contents were determined as bovine serum albumin (=BSA, Sigma, U.S.A.) as a standard protein by Lowry-Folin method with U.V. absorbance at 540 nm[23].

(3) Monosaccharide Analysis: Each fraction (20 mg) and each standard monosaccharide (10 mg) were dissolved in 3% HCl-methanol (2 ml) and methanolyzed at 80±5°C for 20 hours in a cap tube filled with nitrogen gas. The methanolysate was filtered, evaporated and dissolved in pyridine (1 ml). Trimethylsilylation was carried out with 0.2 ml of hexamethyldisilazane and 0.1 ml of trimethylchlorosilane. Under the conditions in Table 1, gas chromatography was performed. Several monosaccharides of the fractions were identified by comparison with retention times of standard monosaccharides. The content of each monosaccharide was calculated from the chromatograms by measuring the peak area (Scheme Ⅵ).

Assay material (20 mg)
- Mixing with 2 ml of 3% HCl-MeOH
- Methanolysis at 80±5°C for 20 hrs.
- Evaporation *in vacuo*
- Addition in MeOH
- Evaporation

Dried methanolysate
- Dissolving in 1 ml of pyridine
- Trimethylsilylation with
  - 0.1 ml of TMCS and
  - 0.2 ml of HMDS

TMS-monosaccharide

G.L.C. analysis

Chromatogram

Scheme Ⅵ. Procedure of monosaccharide analysis

(4) Infrared Spectra: Each fraction (1 mg) was analyzed by KBr disc method.

RESULTS

1) Yield of the Cultured Mycelia

The mycelia obtained from 15 L of culture broth of *V. bombycina* were subjected to hot water extraction and subsequently ethanol precipitation. A brownish powder (2.7 g) designated as Fraction (=Fr.) A was obtained.

2) Purification

Fr. A (1.2 g) was fractionated into three-major peaks by gel filtration on Sepharose 4B (Fig. 1). The yields for Fr. B, C and D were 100, 160 and 80 mg respectively.

3) Antitumor Activity

The antitumor effect of each fraction was assayed by comparing the growth of sarcoma

Table 2. Antitumor effects of the protein-bound polysaccharide fractions of *Volvariella bombycina*

Group	Dose (mg/kg/day, *i.p.*)	Average tumor wt. (g)	Inhibition ratio(%)	Complete regression
Control	Saline	3.50±0.73	—	—
Fr. A	20	1.39±0.33***	60.3	1/9
Control	Saline	5.67±0.20*	—	—
Fr. B	20	1.64±0.38	71.1	0/8**
Fr. C	20	2.60±0.40	54.1	0/8
Fr. D	20	4.06±0.29	28.4	0/7
Fr. E	20	5.23±0.44****	7.8	0/8

*Mean±standard error ***p<0.001
**Numbers of mice used ****N.S.

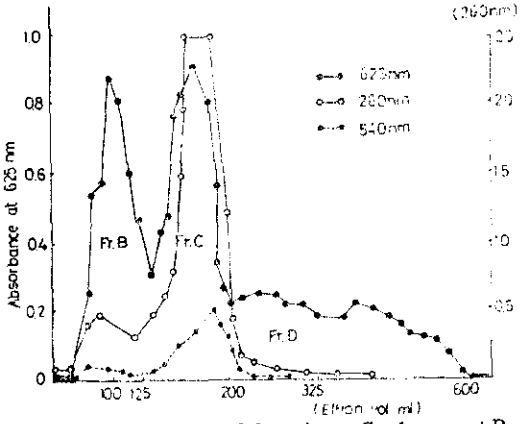


Fig. 1. Separation of Fr. A on Sepharose 4 B.

180 tumor in mice. Each fraction was administered at a dose of 20 mg/kg/mouse/day. The results were shown in Table 2. Of the five fractions tested, Fr. B showed the highest inhibition ratio of 71.1%.

4) Chemical Analysis

The contents of the total polysaccharide and total protein of the fractions were shown in Table 3.

As shown in Table 4 and Fig. 2, the major monosaccharide subunits of the three fractions were glucose, mannose and galactose.

Infrared spectra of the fractions were depicted in Fig. 3.

Table 3. Polysaccharide and protein contents of the antitumor components

	Fr. A	Fr. B	Fr. C
Polysaccharide(%)	39	58	47
Protein(%)	45	19	28

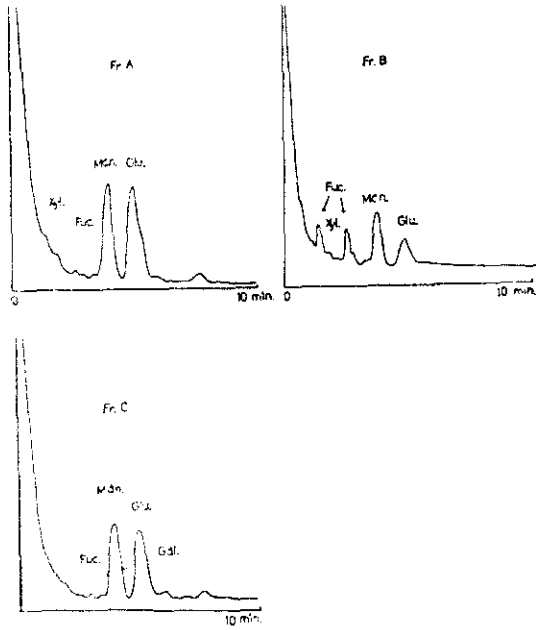


Fig. 2. G.L.C. patterns of the three fractions of the antitumor components *V. bombycina*

5) Effects on Immune Responses

(1) Effects on PEC Population: The counts

Table 4. Monosaccharide contents of the polysaccharide fractions

	Fr. A	Fr. B	Fr. C
Glucose	51(%)	34	45
Fucose	2	17	0.4
Xylose	0.3	19.5	+
Mannose	43	26	38
Galactose	+	+	8

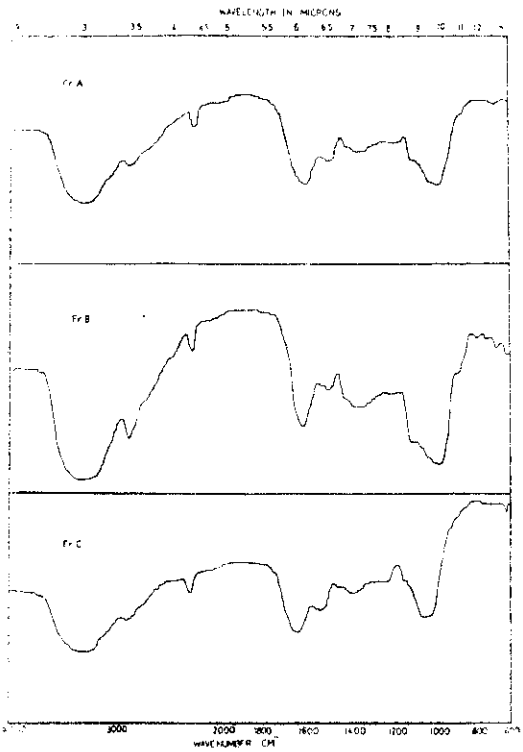


Fig. 3. IR spectra of the antitumor components of *Volvariella bombycina*

of PECs and NSE-positive macrophages showed the maximum on the 24th hour after sample administration and decreased slowly. PMNs also showed the highest count after 24 hours of sample injection. The results of accumulation of cells in the peritoneum were depicted in Fig. 4.

(2) Effects on Plaque-Forming Cells: Non-

Table 5. 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)	33.3±1.6	34.6±3.5
Spleen weight(mg)	166.7±2.9	227.5±3.5
Spleen cell count (1×10^7)	7.5±0.6	12.9±0.3
PFC/10^6 spleen cells	4.7±0.6	82.3±2.2
PFC/spleen($\times10^2$)	3.5±0.5	106.2±2.8

*Mean±standard deviation

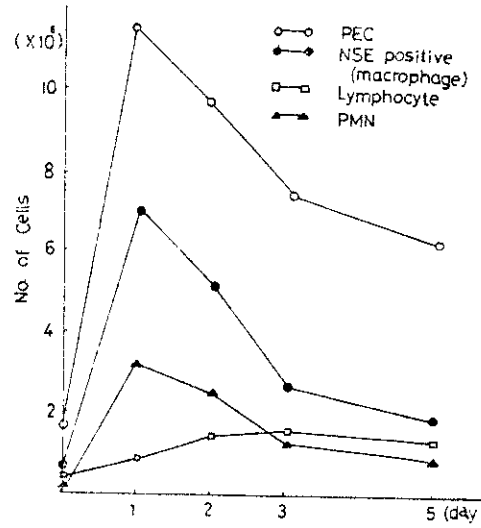


Fig. 4. Effects of Fr. A on the peritoneal cell population of ICR mice

treated control mice produced only a few PFC in their spleens. The mean number of PFC per 10^6 spleen cells was only 4.7. The treated mice, however, showed about seventeen times higher than counts of the control. The result was summarized in Table 5.

DISCUSSION

As described in the results, the protein-polysaccharide obtained from the cultured mycelia of *Volvariella bombycina* showed an

antitumor activity against solid sarcoma 180. But the culture filtrate was ineffective. Especially, Fraction B obtained after the purification showed the highest activity (71.1%).

This fraction contained 58% polysaccharide and 19% protein. The heteroglycan was composed of glucose, mannose, fucose and xylose. In the IR spectra of Fractions A, B and C, 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 the polysaccharides.

Although the structural features of the extracted compounds were not elucidated, the characteristics were deduced from methylation, enzymic degradation[16] and C-nuclear magnetic resonance (NMR) spectroscopy[8,10]. It was reported, however, that lentinan was mainly composed of β-1,3 glucan and that its molecular weight was about 500,000 but that it lacked peptide. PSK was reported to mainly consist of β-1,4 : 1,3 or β-1,4 : 1,6 glucans and 10~15% peptide with a total estimated molecular weight of more than 94,000.

In order to elucidate mechanism of the antitumor activity, effects on immune responses were examined. Peritoneal exudate cells and NSE-positive macrophages reached the maximum on the 24th hour after the administration of the antitumor protein-polysaccharide. Also polymorphonuclear lymphocytes showed their highest counts on the 24th hour. Although the mechanism of its action was not fully understood, it was considered that the effect was through cell-mediated immunity. For more detailed studies on the process of cell-mediated immunity, delayed type hypersensitivity (DTH) reaction will be required.

In addition, the effect of this protein-polysaccharide on the immune response of mice immunized with SRBC was studied.

The protein-polysaccharide was found to potentiate the production of hemolytic plaque-forming cells of spleen. Therefore its antitumor effect was exerted through host-mediated immune mechanism.

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

CONCLUSION

The protein-polysaccharide extracted from the cultured mycelia of *Volvariella bombycina* showed an antitumor activity against sarcoma 180 in ICR mice.

The antitumor components were purified on Sepharose 4 B and separated into three fractions. Of these, Fraction B showed the highest inhibition ratio of 71.1% at a dose of 20 mg/kg/day for 10 days.

Fraction B contained 58% polysaccharide and 19% protein. The polysaccharide moiety of Fraction B consisted of glucose, mannose and galactose.

Fraction B increased peritoneal exudate cells and the accumulation of NSE-positive macrophages. In addition, it also increased plaque-forming cells.

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