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Studies on Antitumor Components of Cultured Basidiomycetes*

Purification and Chemical Analysis of Antineoplastic Constituents of Cultured Mycelia of *Laccaria laccata*

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培養 擔子菌類의 抗癌 成分에 관한 硏究*

애기졸각버섯 培養 菌絲의 抗癌 成分의 精製 및 化學 分析

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Abstract: To produce and characterize antineoplastic constituents in the submerged cultured-mycelia of *Laccaria laccata*, the mycelia were extracted with distilled water. Purification of the extract was carried out by acetone precipitation, by ion exchange chromatography using DEAE-Sephadex A-50, CM-Sephadex C-25 resins, and by gel filtration chromatography on Sephadex G-200. Each fraction obtained during the purification was examined for antineoplastic activity against sarcoma 180 in ICR mice. As the purification proceeded, the antineoplastic activity was markedly increased. The highly purified Fraction E showed 75% tumor inhibition ratio at a dose of 10mg/kg/day and contained 81% polysaccharide and 4% protein. The antitumor component of Fraction E stimulated an accumulation of peritoneal exudate cells including peritoneal macrophages, and is named laccaran.

Keywords: *Laccaria laccata*, Submerged culture, Basidiomycete, Antitumor activity, Protein-polysaccharide, Immunopotentiation, Purification, Total peritoneal cells, Macrophage, PMN, Laccaran.

The constituents of the higher fungi such as antimicrobial, toxic, hallucinogenic, hypocholesterolemic, and antineoplastic components have been investigated continuously. Recently it was reported that polysaccharides from certain higher fungi, for example, lentinan from *Lentinus edodes*, and PS-K from *Coriolus versicolor*, have strong antitumor activities against sarcoma 180 and other tumors (Chihara *et al.*, 1969; Ohno *at al.*, 1975). Also, there is a possibility to use these polysaccharides as immunotherapeutic agents in cancer chemotherapy without causing acute toxicity. Studies on the antitumor activities of protein-bound polysaccharides of Korean higher fungi have been already conducted in our laboratory. (Kim *et al.*,

* Studies on Constituents of the Higher Fungi of Korea (XXXIX). This was presented at the Third International Mycological Congress held at Tokyo, Japan, August 28~September 3, 1983.

1979; Park *et al.*, 1979; Kim *et al.*, 1980; Shim, 1981; Kang *et al.*, 1981; Chung *et al.*, 1983; Kim *et al.*, 1983)

Previously an antitumor polysaccharide fraction of the fruiting bodies of *Laccaria laccata* was reported by us(Kim *et al.*, 1982).

In the present experiments, the antitumor fractions obtained from the cultured mycelia of *L. laccata* were subjected to purification with DEAE-Sephadex, CM-Sephadex, and Sephadex G-200. Their physical and chemical properties and amino acids and monosaccharide components were also elucidated. Especially, to study mechanism of their antitumor activity, effects on immune response were examined.

Materials and Methods

Material

The strain of *Laccaria laccata* ISA-Ll-1008 employed in this work was cordially provided by the Institute of Agricultural Sciences at Suweon in Gyeong-Gi Province, Korea.

Submerged Culture Media

Fifty grams of glucose, 10g peptone, 10g yeast extract, 0.87g KH_2PO_4, 0.5g $MnSO_4 \cdot 7H_2O$, 0.3g $CaCl_2$, 10mg $FeSO_4 \cdot 7H_2O$, 7mg $MnCl_2 \cdot 4H_2O$, 4mg $ZnSO_4 \cdot 7H_2O$, 1mg $CuSO_4 \cdot 5H_2O$. were dissolved in one liter of water. After filtration, it was adjusted to pH 5.0 and autoclaved.

Culture Methods

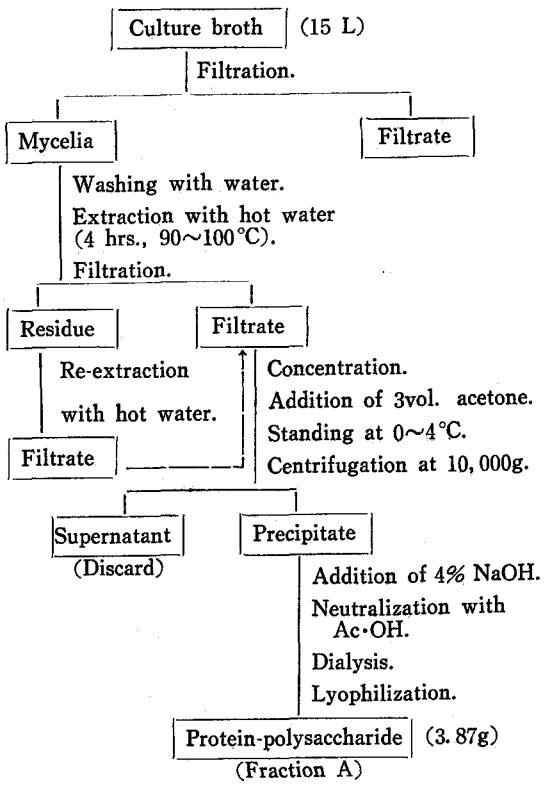
The mycelium of *L. laccata* ISA-Ll-1008 preserved on PDA (Difco Lab.) slant was transferred aseptically to 100 ml of the submerged culture medium and homogenized for 10 seconds in a microblender. For the first submerged culture, 125 ml of the submerged culture medium in a 500 ml flask was inoculated with 10 ml of the mycelial homogenates, and then cultured at 28°C for seven days in an Orbital Shaking Incubator. Again, the fully grown mycelia in the flask were homogenized for 10 seconds and transferred aseptically to 250 ml of the submerged culture medium in a 1*l* flask and cultured in the same condition for seven days.

Extraction of Protein-polysaccharide Fraction

Mycelia obtained from 15 *l* culture broth were filtered and washed with distilled water. The mycelia obtained were homogenized in a blender and subsequent extraction was performed twice on a water bath at 90~100°C for four hours. After filtration, all the filtrates were combined and then concentrated in a vacuum evaporator. Three volumes of acetone were added to the cooled concentrate to avoid denaturation and permitted to stand at 0~4°C overnight in order to complete the precipitation.

The precipitate was collected by centrifugation at 10,000 g for 20 min and dissolved in 450 ml of 4% sodium hydroxide and then the residue was removed. The supernatant was neutralized with concentrated acetic acid to pH 6.0 immediately.

This solution was dialized against distilled water at 4°C for three days using Visking tubes. After dialysis, insoluble materials were discarded by centrifugation at 10,000 g for 20 min, the supernatant



Scheme I. Extraction of protein-polysaccharide fraction from the culture broth of *L. laccata*.

was condensed under reduced pressure and freeze-dried in a lyophilizer. Protein-polysaccharide fraction as odorless and tasteless dark brownish powder was obtained with a yield of 3.87g(Scheme I). This was designated as Fraction A.

Purification of Protein-polysaccharide Fraction

A) DEAE-Sephadex A-50 Column Chromatography

Chromatographic procedure was carried out at 4°C according to the general method of Sober *et al.*(1956). Two grams of preswollen DEAE-Sephadex A-50 (capacity 3.5±meq/g, chloride form) was packed in a column (25×30cm) and equilibrated with 0.01M phosphate buffer. Three grams of Fraction A was first applied to a column and then eluted with 0.01M phosphate buffer (pH 8.3) at a flow rate of 30ml/hr. The non-adsorbent portion was combined. After concentration and dialysis against deionized water, three volumes of ethanol were added and the precipitate was obtained. Thus 1.3g of Fraction B was acquired by lyophilization as light brownish powder.

The adsorbents were separated from the ion exchanger by means of gradient elution system mixture in 0.01 M phosphate buffer(pH 8.3): one chamber filled with 125 ml of 0.25% sodium chloride and the other with 125 ml of 4% sodium chloride.

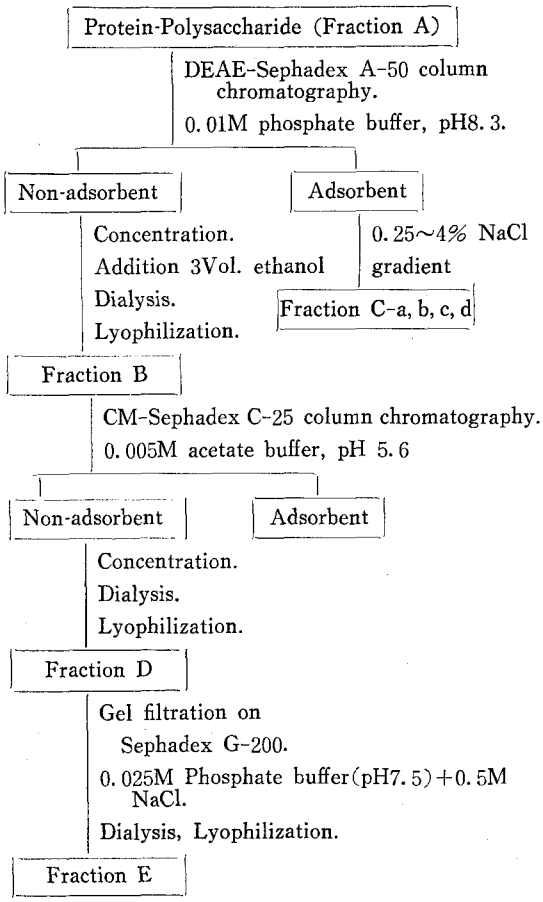
Twenty-ml aliquots of the effluent were collected in a fraction collector. Four peaks were detected by Anthrone test. Each Anthrone-positive peak was treated in the same method as previously mentioned and yielded 81.9 mg of red brownish powder from Fraction C-a, 27.2 mg from Fraction C-b, 5.6 mg from Fraction C-c, and 6 mg from Fraction C-d.

B) CM-Sephadex C-25 Column Chromatography

About one gram of Fraction B was dissolved in 0.005M acetate buffer. This suspension was a collodial solution with high viscosity and did not result in precipitation after prolonged standing. Therefore, the diluted-suspension was applied to a column (1.7×24 cm) packed with CM-Sephadex C-25.

These were subsequently eluted at 4°C with a gradient of 0.25~4% sodium chloride buffered at pH 5.6 at a rate of 10 ml/30 min.

After it was treated in the same manner as previously described, white powder with a yield of 880 mg was gained from the non-adsorbent (Fraction D).



Scheme II. Purification procedures of the protein-polysaccharide of *L. laccata*.

C) Gel Filtration on Sephadex G-200

Sephadex G-200, medium grade, freed of fine particles was allowed to swell for one day in 0.025 M phosphate buffer before being packed in a column.

Seven hundreds mg of fraction D were applied to a column (2.2×70 cm) equilibrated with 0.025 M phosphate buffer (pH 7.5), and eluted with 0.5 M sodium chloride in the same buffer using Mariotte flask at 4°C.

After lyophilization of the dialyzable solution, 254.7 mg of Fraction E was obtained as a white fibrous flake. These purification procedures were summarized in Scheme II.

Antitumor Test

1) Animals

Female ICR strain mice weighing 16~20 g were supplied from the Experimental Animal Farm of Seoul National University.

2) Tumor Cells

Sarcoma 180 cells maintained in the peritoneum of ICR mice with weekly passage in our laboratory were used for testing antitumor activity. The mice were transplanted with sarcoma 180 intraperitoneally. After one week, peritoneal fluid was withdrawn. After washing several times with saline, peritoneal fluid was adjusted to tumor cell number of 1×10^7 cell/ml.

3) Test Solution

To prepare test solutions at a dose of 10mg/kg or 20 mg/kg of the five fractions, 40 mg of Fraction A and 20 mg of Fractions B, C, and D were dissolved in 10 ml of saline. These solutions were autoclaved and stored in a refrigerator.

4) Antitumor Test

In order to test antitumor effects of each fraction, five groups of 10 mice each were inoculated with 0.1 ml of sarcoma cell suspension (1×10^6 cell/0.1ml) into the left groin. Injection of the test solution was initiated intraperitoneally, and continued daily for 10 days starting 24 hours after tumor transplantation.

On the 28th day after tumor transplantation the mice were killed, the tumors were dissected and weighed. The inhibition ratio was calculated according to the following formulus:

$$\text{Tumor Inhition Ratio}(\%) = \left(1 - \frac{Tw}{Cw}\right) \times 100$$

Tw=Average tumor weight of treated group

Cw=Average tumor weight of control group

Effects of Fraction E on Peritoneal Cell Population

1) Materials

(1) Animals

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

(2) Tumor Cells

Sarcoma 180 cells were collected from the ascites of four tumor-bearing mice. After washing several times with saline, sarcoma suspension was sonicated for five minutes. Blood cells were removed with 0.83 % ammonium chloride.

The sonicated cells were dialyzed against distilled water at 4°C for three days. The precipitate was discarded by centrifugation, and supernatants were inoculated subcutaneously into the left groin of the mice.

2) Methods

In this experiment, 54 mice were divided into three groups : a soluble starch grup as a control, a stimulated group injeted with 1 mg/ml of Fraction E and a sonicated tumor-transplanted group which was injected with 1 mg/ml of Fraction E. Injection volume was one ml for each mouse only one time. Fraction E and soluble starch were dissolved in 0.1 M phosphate-buffered saline at pH7.0 and injected intraperitoneally. The solutions were autoclaved before use.

(1) Total Cell Count (PEC Count)

Three mice in each group were sacrificed at differ-er.t intervals (5 hrs., 1 day, 2 days, 3 days, and 5 days) after injection. The accumulated cells in the peritoneal cavity were washed with BSS vigorously. The total cells were directly counted by using a haemacytometer

(2) Macrophage Count(Nonspecific Esterase Stain)

This method is dependent upon demonstration of macrophage enzymes, *i. e.*, nonspecific esterases, the most reliable cytochemical macrophage identification (Philipp, 1981).

The specimen on a glass slide was air-dried, fixed in cold acetone for five min, and incubated in substrate at 37°C for 30 minutes. After the slide was washed in tap water, it was counterstained with 1% methylgreen and mounted in glycerol jelly. The cells which contained macrophage were identified as a reddish granule.

(3) PMN Count (Giemsa Stain)

To count polymorphornucleoleucocytes (PMN), the specimen on a glass slide was air-dried and fixed in absolute methanol for five minutes.

The slide with freshly prepared Giemsa stain was covered for 25 minutes. The slide was rinsed in 95% ethanol to remove any excess dye and examined

examined. The cells which contained a multi-lobbed or doughnut-type nucleus was counted as PMN (Abdul, 1980).

Chemical Analysis

1) Total Polysaccharide Content

Total polysaccharide content of each fraction was quantitatively determined by Anthrone test using glucose as a standard sugar. The degree of absorption was measured by U.V. spectrophotometer at 625 nm after Anthrone test and the polysaccharide content was calculated from the calibration curve.

2) Monosaccharide Contents

Twenty milligrams of each fraction and 10 mg of each standard monosaccharide were dissolved in two ml of 0.75 N hydrochloric acid-methanol in cap tubes. Methanolysis was performed at 80±5°C for 30 hours. The methanolysate was filtered, evaporated and dissolved in one ml of pyridine. Trimethylsilylation was carried out using 0.1 ml hexamethyldisilazane and 0.1 ml of trimethylchlorosilane. Under the condition in Table I gas chromatography was performed. Several monosaccharides of the fractions were identified by comparison with retention times of authentic sugars. The content of each monosaccharide was calculated from the chromatograms by measuring the peak area.

3) Total Protein Content

Protein content of each fraction was measured by Lowry-Folin test using bovine serum albumin as a standard protein with U.V. spectrophotometer at 750 nm, and was calculated from the calibration curve.

4) Amino Acid Contents

Twenty milligrams of each fraction were dissolved in five ml of 6N hydrochloric acid in ampules. The ampules were filled with nitrogen gas in order to prevent oxidation and then sealed. Hydrolysis was performed at 110±5°C for 20 hours. The hydrolysates were filtered, evaporated and dissolved in 2 ml of 0.02 N hydrochloric acid. Amino acids of each fraction were identified by comparison with the chromatogram of standard amino acids, and the quantity of each amino acid was calculated from the chromatograms by peak height method.

Table I. Measurement Condition of G.L.C.

Column	3% OV-17 (80-100 mesh shimalite)	
	3mm×1m borosilicate glass column	
Temperature	column	:160°C
	detector	:190°C
	injector	:190°C
Flow rate	N_2	:50ml/min
	H_2	:60ml/min(0.8kg/cm²)
	Air	:88ml/min (1.2kg/cm²)

Results

Yield of the Cultured-mycelia

Twenty two and half grams of the mycelia were obtained from 15*l* of culture broth of *L. laccata*.

Antitumor Activity

Of the five farctions tested, Fraction E showed 75.0 % tumor inhibition ratio at a dose of 10 mg/kg/day, which was most effective, and complete regression was observed in three out of nine mice at the end of 28 days after the tumor transplantation. Table II

Table II. Antitumor effects of the five fractions on sarcoma 180 in mice.

Group	Dose (mg/kg/day)	Number of mice	Average tumor weight(g)	Inhibition ratio(%)	Complete regression
Control	Saline	10	4.03±1.23*	—	—
Fraction A	20	9	1.58±0.75	60.9	1
Fraction B	10	9	1.46±0.57	64.0	2
Fraction C	10	9	1.50±0.61	62.5	—
Fraction D	10	9	1.26±0.49	68.5	2
Fraction E	10	9	1.00±0.35	75.0	3

*The values are means±standard error.

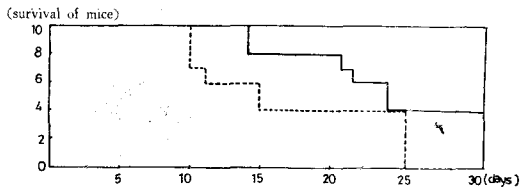


Fig. 1. Effects of fraction E on the life span after intraperitoneal transplantation of sarcoma 180 in mice (5×10^4 cell/mouse).
····· : Control and —— : 10mg/kg/day

exhibits the antitumor activity. Fig. 1 displays the life span of the treated group which was longer than that of the control group.

Chemical Composition of Five Fractions

1) Contents of the Polysaccharide Moiety and its Monosaccharides

As shown in Table III, total polysaccharide contents by Anthrone test and the contents of each monosaccharide by G.L.C. were measured. The major monosaccharide subunits of five fractions were glucose, mannose and galactose.

Table III. Polysaccharide and monosaccharide contents of the five fractions.

Fraction		A	B	C	D	E
Total Polysaccharide content*		55.0(%)	70.5	51.0	77.0	81.0
Monosaccharide contents* :	Fucose	2.6	4.1	4.8	4.2	6.5
	Xylose	1.8	3.1	3.8	3.3	5.0
	Mannose	42.1	24.6	31.4	28.3	36.0
	Galactose	17.5	27.7	—	26.7	23.7
	Glucose	36.0	40.5	60.6	37.5	28.8

* Percentage to the protein-polysaccharide component
** Percentage to polysaccharide fraction of the protein-polysaccharide component.

Table IV. Protein contents of the five fractions.

Fraction	A	B	C	D	E
Total protein*	21.0(%)	6.0	18.0	4.0	4.0

* Percentage to the protein-polysaccharide component.

Table V. Amino acid contents of fractions D and E.

Amino acid content*	Fraction D	Fraction E
Trp	24.8%	7.6%
Lys	5.8	7.3
His	8.0	9.4
Arg	20.3	3.6
Thr	1.9	2.8
Ser	4.4	4.5
Glu	8.4	11.3
Pro	0.6	0.8
Gly	2.9	3.3
Ala	3.5	6.8
Val	10.9	22.5
Met	0.3	0.7
Ile	1.0	2.3
Leu	1.8	5.4
Tyr	0.8	0.9
Phe	3.2	3.6
Asp	1.5	7.2

* Percentage to the protein fraction of the protein-polysaccharide component.

2) Contents of the Protein Moiety and its Amino Acids

Tables IV and V exhibit the total protein contents by Lowry-Folin method and amino acid contents by automatic amino acid analyzer. The major amino acids of Fractions D and E were tryptophane and valine, respectively.

Separation and Purification of the Protein-bound Polysaccharide

Fig. 2 depicts the eluate of Fraction C chromatographed on DEAE-Sephadex A-50 with the buffered-gradient salt solution mixture. Four Anthrone-positive peaks which coincided with U.V. absorption at 260 nm were obtained accurately.

Gel filtration on Sephadex G-200 was shown in Fig. 3. Being eluted near the void volume, only one

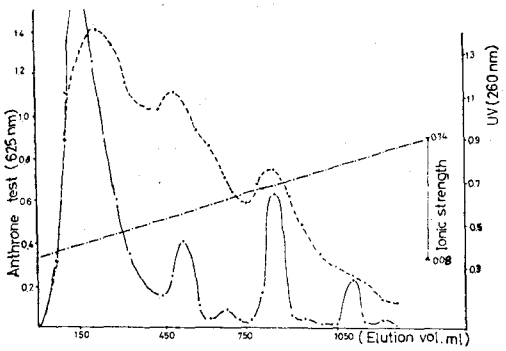


Fig. 2. DEAE-Sephadex A-50 column chromatography of Fraction C.

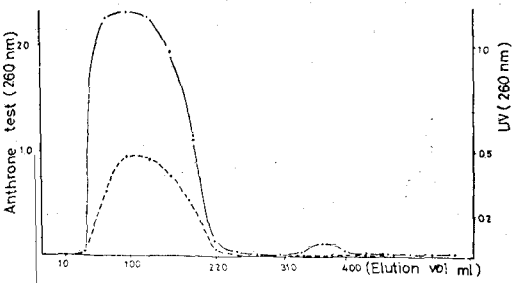


Fig. 3. Gel filtration on Sephadex G-200 of Fraction E.

peak seems to be homogeneous and an assemblage of large molecular weight. Moreover this peak coincided with U.V. absorption at 260 nm and this result proved that Fraction E contained a polysaccharide combined with a small amount of a protein.

IR Spectra of the Antitumor Component

IR spectra showed no characteristic absorption except C-H, O-H and C-O vibration in Fractions A and E. As compared with the IR spectrum of Fraction A, that of Fraction E was slightly changed in spite of purification with ion exchange chromatography and gel filtration (Fig. 4).

Effects of the Antitumor Component on Immune Response

There was an increase in number of accumulated cells in peritoneal cavity of the Fraction E-injected mice(Fig. 5). This phenomenon is similar to that observed by Diller *et al.* in response to yeast polysaccharide(Diller *et al.* 1963). Although the mechanism of antitumor activity of Fraction E is not fully understood, a host-mediated antitumor activity can be suggested as a part of its mechanism.

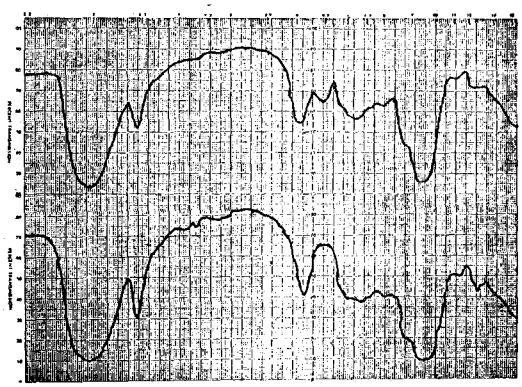


Fig. 4. IR spectrum of the Fraction A(above) and Fraction E(below) in KBr.

Disscussion

In our previous report (Kim, S.H. *et al.*, 1982), the protein-polysaccharide extracted from the carpophores of *Laccaria laccata* showed a tumor inhibition ratio of 75% against sarcoma 180 in ICR strain mice. We also reported that the mycelia of this edible mushroom were grown in submerged culture in an artificial medium and that they produced the protein-polysaccharide which showed 58% ratio in the tumor inhibition. As the results of the present study showed, antitumor activity of the cultured-mycelia of *Laccaria laccata* was analogous to that of the carpophores. These facts present a potentiality for production of the

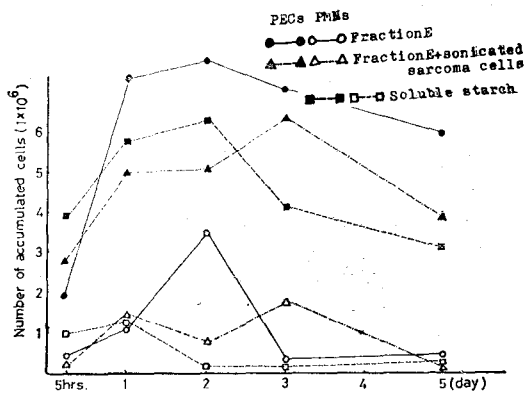


Fig. 5. Cell accumulation in the peritoneal cavities of three groups.

antitumor component by the culture of the mycelia. Purified Fraction E showed the highest activity. However, it seems less active than lentinan of *Lentinus edodes* (Chihara *et al.*, 1969) and PSK of *Coriolus versicolor*, (Ito *et al.*, 1972) although the data are not directly comparable.

The antitumor protein-polysaccharide of this mushroom may be unique in that its prevalent monosaccharides were mannose, glucose and galactose, whereas those of other antitumor polysaccharides were galactose and glucose in the case of *Ramaria formosa*(Yoo, *et al.*, 1982), fucose in that of *Leatiporus sulphureus* (Kang *et al.*. 1982), glucose in that of *Trametes sanguinea* (Hong *et al.*, 1982). The major monosaccharide pattern of this fungus is somewhat similar to those of *Cryptoporous volvatus* (Kim, B.K. *et al.*, 1982), and *Armillariella mellea* (Kim, J.S. *et al.*, 1983), and yet the ratio of the monosaccharide content was different from those of the latter two components. It is also likely that the polymerization sequence of these monosaccharides and the three-dimensional structure of the polymer may vary considerably depending upon the species of the fungi. It is of interest that all immunoglobulins contain 3~12% carbohydrate attached to globulin and that it consists of mannose, galactose, fucose, N-acetylglucosamine and N-glycolylneuraminic acid(Brenckle and Kornfeld, 1980).

In the separation and purification and of the protein-polysaccharide, an increase in its antitumor activity after the purification with ion exchange and Sephadex G-200 was associated with an increase in the ratio of the polysaccharide content with a corresponding decrease in the protein content. This result indicates that the amount of polysaccharide moiety was of primary importance for the antitumor activity.

Although the experiments on the effect of Fraction E on immune response of total cells and macrophages gave rather controversial results, the antitumor component is considered to be an excellent immuno-accelerator of the cell-mediated immunity and, therefore, is named laccaran.

On the contrary to the severe toxicities of the current antineoplastic chemotherapeutics, this antitumor fraction did not exhibit any acute toxicity in the mice.

Further correlation of the immune response and the antitumor activity will require a more detailed study of the immunological properties of laccaran of *L. laccata.*

Conclusions

The protein-bound polysaccharide extracted from the cultured mycelia of *Laccaria* showed an antitumor activity against sarcoma 180 in mice. The antitumor components were purified and separated into five fractions. Of these, the highly purified Fraction E contained 81% polysaccharide and 4% protein. With the exception of Fraction C, the polysaccharides of four fractions consisted of glucose, galactose, mannose, xylose, and fucose. The protein of Fractions C and D contained 17 amino acids respectively. The antitumor component of Fraction E the production of PECs and macrophages in mice. This component is named laccaran.

Acknowledgments

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This report is dedicated to the late Professor Young Eun Kim, a distinguished scholar and teacher, who rendered us his constant encouragement and advice to our research.

적 요

애기졸각버섯의 균사를 액내 진탕 배양법으로 배양하여 항종양성 성분을 생성시키고 이 성분의 특징을 밝히기위하여 위의 방법으로 배양된 균사체를 증류수로 가온 추출하였으며 그 추출물을 이온 교환수지 및 겔여과크로마토그라피법으로 정제하였다. 이 정제과정에서 분리된 5개의 분획에 대하여 각각 항종양 실험을 시행하였던 바, 그 정제의 정도에 비례하여 항암작용이 증

가하였으며, 최종 분획 E를 1회 10mg/kg용량으로 백서에 투여하였을 때 75%의 종양 억제율을 나타내었으며, 다당류 81% 및 단백질 4%를 함유하고 있었다. 이 성분은 백서의 복강내 마크로훼이지의 수를 증가시켰으며 이 성분을 laccaran으로 명명한다.

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