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Studies on Constituents of Higher Fungi of Korea(XXXV)

Antitumor Components Extracted from the Carpophores and Cultured Mycelia of *Laccaria laccata*

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韓國産 高等 菌類의 成分 硏究(第35報)

애기졸각버섯의 抗癌 成分

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Abstract: To find antitumor components, a protein-bound polysaccharide fraction was obtained by extracting with hot water and precipitating with ethanol from the carpophores and the cultured mycelia of *Laccaria laccata*. The fraction was examined for antitumor activity against sarcoma 180 implanted in mice. The component extracted from the carpophores showed 75% and 65% tumor inhibition ratios in the doses of 20 mg and 50 mg/kg/day, respectively. The protein-bound polysaccharide fraction of the cultured mycelia of *L. laccata* also showed 57.8% tumor inhibition ratio in the dose of 20 mg/kg/day. The chemical analysis of the protein-bound polysaccharide fraction showed that it contained a polysaccharide and a protein. The hydrolysates of the polysaccharide moiety contained six and one unknown monosaccharides. Fourteen amino acids were identified in the hydrolysates of the protein moiety.

Introduction

Numerous investigations concerning the constituents of the higher fungi such as antimicrobial, toxic, hallucinogenic, hypocholesterolemic, antineoplastic constituents and trace elements have been made. Especially, studies on antitumor components of higher fungi have been extensively carried out (Tsukagoshi & Ohashi, 1974; Ito *et al.*, 1977; Ikekawa *et al.*, 1973). They were concerned with fractionation and purification of antitumor constituent, determination of chemical structure, mechanism of antineoplastic activity, adjuvant effect with immunosuppressive agent, antiviral activity and condition of fermentation (Homa & Kuratsuke, 1973; Sasaki & Takasuka, 1976; Fuji *et al.*, 1978; Luzio *et al.*, 1978; Miyazaki *et al.*, 1979; Nakashima *et al.*, 1979; Iizuka, 1980; Usui *et al.*, 1981).

For the past decade, our laboratory has continuously reported on the constituents of Korean higher fungi. We have isolated antitumor protein-bound polysaccharides from the carpophores and cultured mycelia of Korean Basidiomycetes to find out stronger antitumor components with a lower toxicity from natural resources. We also elucidated the chemical characteristics and the antitumor activity mostly against sarcoma 180 in mice (Kim *et al.*,

1973; Kim *et al.*, 1976; Kim *et al.*, 1977; Kim *et al.*, 1978; Kim *et al.*, 1979; Park *et al.*, 1979; Park *et al.*, 1979; Kim *et al.*, 1980; Shim, 1981; Kim *et al.*, 1982).

In the present study, the antitumor activity of the protein-bound polysaccharide fractions from the carpophores and cultured mycelia of *Laccaria laccata* against sarcoma 180 implanted in mice was examined and the possibility of mass production of the antitumor component from cultured mycelia was also tested. In addition, the chemical assay of the protein-bound polysaccharide was performed.

While there have been several reports on the mineral elements, acid phosphatase activity and amino acids of *Laccaria laccata*, no reports have been published on the antitumor activity (Andrzej *et al.*, 1967; Haselwandter, 1978; Dziadowiec, 1979; Abe *et al.*, 1980).

Materials and Methods

Experiments on the Carpophores

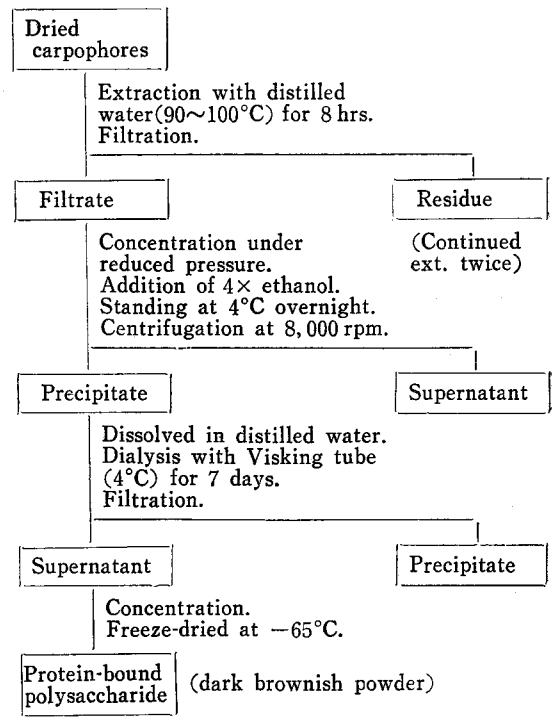
1) Carpophores

Laccaria laccata (Fr.) Berk. *et* Br. belongs to the family *Tricholomataceae* and has convex pileus with a little depression in the center, 1.5~2cm or more broad. Its spores are white, elliptical and aculeate. It grows in humid woods, grass, and moss, especially under poplars from summer to autumn. It is edible (Lee *et al.*, 1959).

The carpophores of *Laccaria laccata* used in this experiment were collected at Suwon in Gyeong-Gi Province in September, 1981. Some of materials are preserved as the specimen in our laboratory.

2) Extraction and Preparation

The carpophores of *L. laccata* were air-dried and broken into small pieces. Fifty grams of the dried carpophores were homogenized in a Waring blender. Extraction with 2,000ml of distilled water was performed by refluxing on a water bath at 90~100°C for 8 hours. After filtration, the residue was extracted with 1,000ml of distilled water under the same condition. All the filtrates were combined and then concentrated to 600ml under reduced pressure. The concentrate was mixed with four volumes of 96% ethanol and allowed to stand at 4°C overnight. The precipitate was centrifugated for 30 minutes at 20°C (Beckman Model J-21) and redissolved for lyophilization. The lyophilized powder was then dissolved in 200ml of distilled water and dialyzed against distilled water at 4°C for 7 days using Visking tubes. After dialysis, insoluble materials were removed and the filtrate was concentrated under reduced pressure. The concentrate was freeze-dried at −65°C in a lyophilized (Edwards high vacuum Model EF03). A protein-bound polysaccharide fraction as an odorless and tastless dark brownish power yielding 1.2g was obtained(Scheme I).



Scheme I. Preparation of the protein-bound polysaccharide from the carpophores of *L. laccata*.

Experiments on the Cultured Mycelia

1) Mycelia

The mycelia of *L. laccata* ISA-L1-1008 used in this experiment were kindly supplied from the Office of Rural Development at Suwon in Gyeong-Gi Pro-

vince.

2) Composition of Medium

A) PDA Slant: Bacto potato, dextrose, agar(Difco Lab. U.S.A.) 39g/1*l*. Autoclaving at 121°C, 15lb., for 20 min.

B) Submerged Culture Medium: Glucose 50g, peptone 10g, yeast ext. 10g, KH_3PO_4 0.87g, $MgSO_4 \cdot 7H_2O$ 0.5g, $CaCl_2$ 0.3g, $FeSO_4 \cdot 7H_2O$ 10mg, $MnCl_2 \cdot 4H_2O$ 7mg, $ZnSO_4 \cdot 7H_2O$ 4mg, $CuSO_4 \cdot 5H_2O$ 1mg per 1*l*. After filtration, the medium was adjusted to pH 5.0 and sterilized at 121°C, 15lb., for 20 min.

3) Culture

L. laccata ISA-L1-1008 maintained on PDA slant was transferred aseptically and homogenized for 10 seconds in a microblender. For first submerged culture 100ml of submerged culture medium in a 500ml flask was inoculated with the above mycelia and then incubated for 7 days on an Orbital Shaking Incubator (Gallenkamp) at 26~28°C and 180rpm. The contents of the flask were homogenized for 10 seconds and transferred aseptically to 100ml of submerged culture medium in a 500ml flask and propagated in the same method (Norris, 1971).

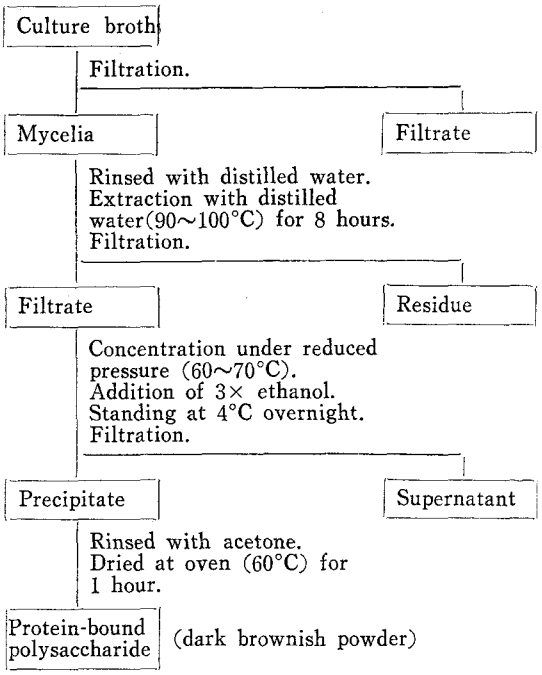
4) Extraction and Preparation

The mycelia from 400ml of the culture grown for 7 days were collected on a Buchner funnel and washed three times with distilled water. The mycelia obtained were homogenized in a microblender and subsequent extraction was carried out two times on a water bath at 90~100°C for 8 hours. After filtration, all the filtrates were combined and then concentrated in a rotary vacuum evaporator. Three volumes of 96% ethanol were added to the concentrate and allowed to stand at 4°C overnight. The precipitate was collected on a Buchner funnel and washed with acetone. After drying in an oven at 60°C for 1 hour, the protein-bound polysaccharide was obtained as a dark brownish powder yielding 150mg (Scheme II).

Antitumor Test

1) Animals

ICR strain mice of male sex weighing 20~25g were used for the experiments. They were supplied from the Experimental Animal Farm of Seoul National University.



Scheme II. Preparation of protein-bound polysaccharide from the cultured mycelia of *L. laccata*.

2) Tumors

Sarcoma 180 cells maintained continuously in our laboratory were used for testing the antitumor activity *in vivo*. Sarcoma 180 cells were intraperitoneally implanted in mice. After one week, the animals were killed and sarcoma 180 ascitic fluid was withdrawn. After washing three times with saline, ascitic fluid was diluted to adjust the tumor cell concentration to 1×10^7 cell/ml.

3) Preparation of Test Solutions

To prepare test solutions for a dose of 20mg/Kg, 40mg of the fraction which were obtained from the carpophores and cultured mycelia of *L. laccata* were dissolved in 10ml of saline. Also 100mg of the fraction were dissolved in 10ml of saline for a dose of 50mg/kg and physiological saline was used for control. These solutions were autoclaved at 121°C, 15 lb, for 20 min and stored in a refrigerator.

4) Antitumor Test

Sarcoma 180 cells (1×10^6 cells/0.1ml/mouse) obtained from ascites of the implanted mice were

inoculated subcutaneously into the left groin of 30 mice. Thirty mice were divided into three groups: a control group and two treated groups. The latter groups received test samples of 20mg or 50mg/kg/day dose intraperitoneally in a volume of 0.1 ml/mouse basis on 24 hours after the tumor cell inoculation, and then, once daily for 10 consecutive days. To the control group 0.1ml/mouse of saline solution was injected in the same manner as the above. Tumor weights were measured on the 30th day after implantation and inhibition ratio was determined by compairson with the tumor weights of the control mice. Tumor inhibition ratio as index of anticancer activity was calculated as follows:

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

Tw=Average tumor weight of the treated group

Cw=Average tumor weight of the control group

Complete regression was also examined on the 30th day after tumor implantation.

Assay for the Protein-bound Polysaccharide

1) Chemical Analysis

The concentration of test solutions containing the protein-bound polysaccharide of *L. laccata* used in these color reactions was 1% (w/v) in all cases. Anthrone test, Molish test, Iodine test, Xanthoprotein test, Tryptophan test, Biuret test, Ninhydrin test, Ninhydrin test on hydrolysate, and Lowry-Folin test were carried out according to the methods described in our previous report (Park *et al.*, 1979; Kim *et al.*, 1979).

2) Analysis of Polysaccharide Moiety

A) Total Polysaccharide Content

Polysaccharide content of the antitumor fraction was determined by the anthrone method using glucose as a standard sugar. The degree of absorption was measured by U.V. spectrophotometer (Unicam SP 1805) at 625nm. Polysaccharide content was calculated from the calibration curve.

B) Monosaccharide Analysis

To determine the identity and content of monosaccharides, 20mg of the polysaccharide and 10mg of each standard monosaccharide were respectively dissolved in 2ml of 0.75N hydrochloric acid-methanol in cap tubes. Methanolysis was carried out at 80±5°C for 20 hours. After filtration, the filtrate was evaporated and dissolved in one ml of pyridine. Trimethylsilylation was carried out using 0.2ml of hexamethyldisilazane and 0.1ml of trimethylchlorosilane. After stirring vigorously for 30 seconds, gas liquid chromatography was performed under the condition in Table I. By comparison with retention time of authentic standard monosaccharides, several monosaccharides of the polysaccharide were identified. The content of each monosaccharide was calculated from the chromatograms by half width and planimetry.

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

Column	1.5% OV-1 (80~100 mesh shimalite) 4mm ID×1.5m boronsilicate 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)

3) Analysis of Protein Moiety

A) Total Protein Content

Protein content of the antitumor fraction was measured by Lowry-Folin method using albumin as a standard protein. The degree of absorption was determined by U.V. spectrophotometer (Unicam SP 1805) at 750nm. Protein content was calculated from the calibration curve.

B) Amino Acid Analysis

To determine the identity and content of amino acids, 20mg of the antitumor fraction were dissolved in 5 ml of 6N-hydrochloric acid in ampules. The ampules were filled with nitrogen gas in order to prevent oxidation and sealed. Hydrolysis was carried out at 110±5°C for 20 hours. After filtration, the filtrate was evaporated and dissolved in 2ml of 0.02N-hydrochloric acid solution. Amino acids were prepared by adjusting their concentration to 3 nmol/50 μl and also analyzed under the same condition. Several amino acids of the fraction were

Table II. Measurement conditions of amino acid analyzer.

Column	2.6×150mm
Ion exchange resin	#2619 (Hitachi)
Flow rate	Buffer soln. 0.225ml/min
	Ninhydrin 0.3ml/min
Analysis cycle time	70 min
Column pressure	80~130kg/cm^2
Ninhydrin pressure	15~35kg/cm^2
Column temp.	53°C
N_2 gas pressure	0.28kg/cm^2
Reaction bath temp.	98°C
Wave length	570nm, 440nm

identified by comparison with the chromatogram of the standard amino acids. The content of each amino acid was calculated from chromatograms by peak height method.

Results

Antitumor Activity

Antitumor activity of the protein-bound polysaccharide from *L. laccata* on sarcoma 180 in mice was shown in Table III. Fig. 1 depicted the effect of the fraction on survival of mice bearing sarcoma 180. The life span of the treated group was longer than that of the control group.

Assay for the Protein-bound Polysaccharide Fraction

1) Chemical Analysis

The results of color reaction were summerized in Table IV. They suggest that the major constituents of the fraction contain a polysaccharide and a

Table III. Antitumor effects of the protein-bound polysaccharide fraction from the carpophores and the cultured mycelia of *Laccaria laccata* on sarcoma 180 in mice.

	Dose (mg/kg/day, *i.p.*)	No. of mice	Average tumor weight (g)	Inhibition ratio (%)	Complete regression
Control	saline	10 ♂	2.20±0.53***		
Carpophores*	20 mg	10 ♂	0.55±0.27 ($p<0.001$)	75	5
	50 mg	10 ♂	0.77±0.49 ($p<0.01$)	65	4
Control	saline	9 ♀	4.82±1.39		
Cultured Mycelia**	20 mg	9 ♀	2.03±0.63 ($p<0.01$)	5.8	1

* mice (20~25g,) were inoculated subcutaneously with sarcoma 180 (5×10^5 cell/mouse).
** mice (18~229,) were inoculated subcutaneously with sarcoma 180 (1×10^6 cell/mouse).
*** values are means±standad error.

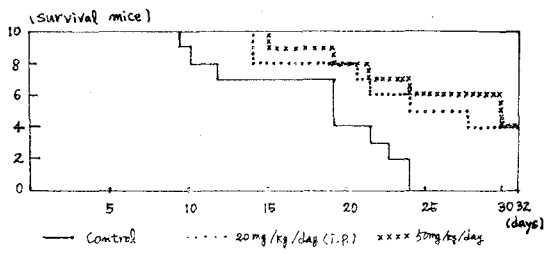


Fig. 1. Effects of the antitumor fraction of *L. laccata* on the life span after intraperitoneal implantation of sarcoma 180 in mice.

Table IV. The various color reactions on the antitumor fraction of the carpophores of *L. laccata*.

Test	Result	
Anthrone test	green	+
Molish test	purple	++
Iodine test		−
Xanthoprotein test	yellow	+
Tryptophan test	violet-brown	++
Biuret test	purple-blue	+
Ninhydin test	blue-violet	+
Ninhydrin test on hydrolysate	violet	++
Lowry-Folin test	dark blue	+

Table V. The polysaccharide and the monosaccharide contents of *L. laccata.*

	Content (%)
Total polysaccharide	33.8
Monosaccharide:	
Fructose	trace
Fucose	7.1
Xylose	4.4
Unknown	3.5
Mannose	15.9
Galactose	19.5
Glucose	49.5

Table VI. Retention times of the TMS-monosaccharides by G.L.C.

TMS-monosaccharide	Retention time
Fucose	2.97, 3.3, 3.6
Xylose	3.94, 4.3
Unknown	5.4
Mannose	6.8
Galactose	7.97, 9.07
Glucose	10, 1104

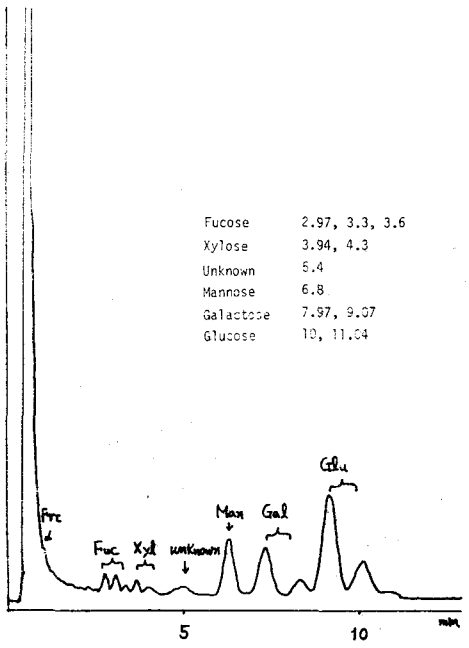


Fig. 2. G.L.C. pattern of the monosaccharides of the antitumor fraction of the carpophores *L. laccata.*

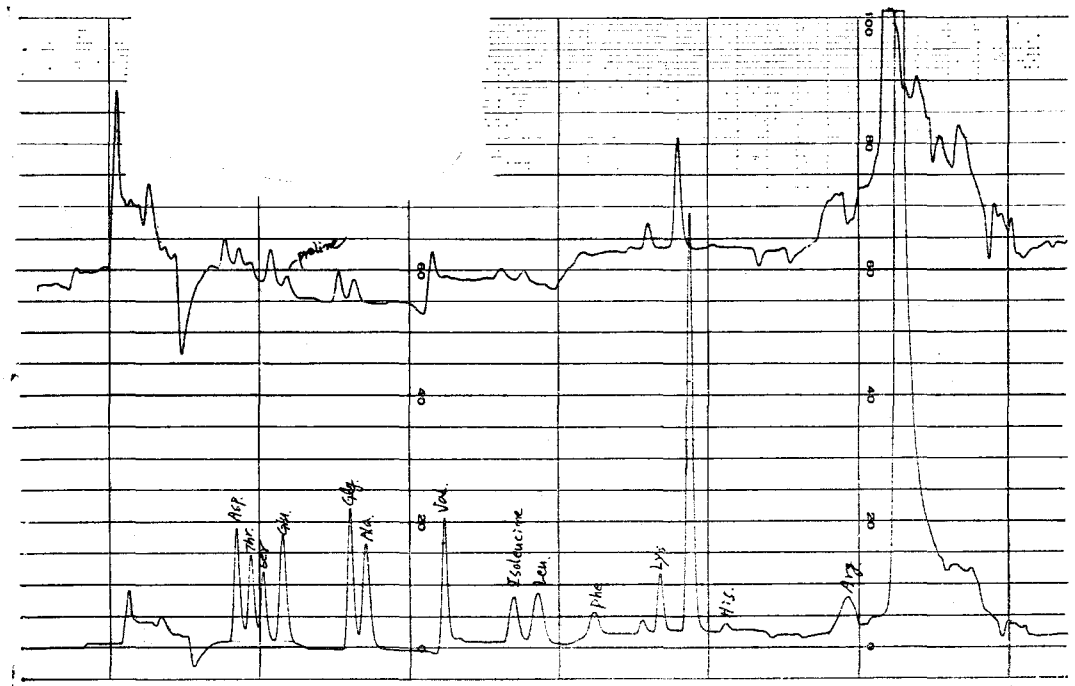


Fig. 3. Chromatogram of amino acids of protein moiety of the antitumor fraction extracted from the carpophores of *L. laccata.*

protein.

2) Analysis of the Polysaccharide Moiety of the Fraction

Tables V and VI showed the total polysaccharide content by anthrone test and the content of each monosaccharide. Fig. 2 showed the G.L.C. pattern of the monosaccharides.

3) Analysis of the Protein Moiety of the Fraction

Table VII showed the total protein content by Lowry-Folin method. The content of each amino acids and that of the protein moiety of the antitumor fraction by automatic amino acid analyzer were shown in Fig. 3.

Table VII. The protein and the amino acid contents of the antitumor fraction of *L. laccata*.

	Content (%)
Total Protein	39.0
Amino Acid	
Asp.	9.70
Thr.	7.19
Ser.	5.63
Glu.	10.01
Pro.	0.25
Gly.	11.88
Ala.	10.94
Val.	13.45
Ile.	5.00
Leu.	6.57
Phe.	2.9
Lys.	8.13
His.	15.63
Arg.	7.50

* Ammonia was also detected.

Discussion

The results showed that the protein-bound polysaccharide fraction of *Laccaria laccata* had a relatively high antitumor activity. That is, the fraction of the carpophores showed 75 and 65% tumor inhibition ratios in the doses of 20 and 50 mg/kg/day respectively. The fraction of the cultured mycelia of *L. laccata* also exhibited 58% tumor inhibition ratio in the dose of 20 mg/kg/day. These facts indicate a possibility of mass production of the antitumor component from the cultured mycelia.

The chemical analysis of the antitumor fraction showed that it contained a polysaccharide(33.8%) and a protain(39.0%) and that the polysaccharide moiety consisted of seven monosaccharides The latter fact suggests that the polysaccharide may be a unique one, since the polysaccharides of other antitumor basidiomycetes contained four monosaccharides in the cases of *Coriolus versicolor* (Park *et al.*, 1979), *Russula pseudodelica* and *Microporus affinis* (Min *et al.*, 1980), *Ganoderma lucidum* (Kim *et al.*, 1980), *Auricularia auricula-judae* (Lee *et al.*, 1981), *Cryptoporus volvatus* (Kim *et al.*, 1982), and five monosaccharides in *Naematoloma fasciculare* (Lee *et al.*, 1981). Hence further chemical studies on this antitumor fraction are warranted.

Since the yield of the antitumor fraction was relatively less in the submerged culture than those of the previous studies (Park *et al.*, 1979; Kang *et al.*, 1981; Shim, 1981), improvements of the productivity and culture conditions of the fungal strain appear to be necessary for adequate production of the fraction.

It was previously reported that the antitumor activity of these protein-bound polysaccharides of the polypores and other basidiomycetes is due to the potentiation of cell-mediated immunty against the tumor (Kang *et al.*, 1981; Maeda & Chihara, 1973; Shim, 1981). It is likely, therefore, that the antitumor fraction of this edible fungus may enhance immunity against the tumor, since no apparent acute toxicity was observed in the organs of the mice. It is of interest to pursue influences of the fraction on tumor immunity.

In summary the protein-bound polysaccharide fraction of *Laccaria laccata* showed an inhibitory activity against sarcoma 180 in mice.

Conclusions

The protein-bound polysaccharide fraction extrac-

ted from the carpophores of *Laccaria laccata* in the doses of 20 and 50mg/kg/day inhibited the growth of sarcoma 180 in mice. The tumor inhition ratios were 75% and 65% respectively. The protein-bound polysaccharide fraction isolated fiom the cultured mycelia of *L. laccata* also showed 58% tumor inhibition ratio in the dose of 20mg/kg/day. The results of chemical analysis showed that the anti-tumor fraction consisted of a polysaccharide and a protein. Six and one unknown monosaccharides were detected in the polysaccharide moiety, and fourteen amino acids were identified in the protein moiety.

Acknowledgments

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요 약

보다 항암력이 강하고 독성이 적은 항암물질을 얻기 위해, 경기도 수원에서 채집한 애기졸각버섯 *Laccaaria laccate*의 자실체 및 배양균사체로 부터 다당체를 분리하여 그 화학적 조성 및 sarcoma 180에 대한 항암효과를 검토하였다.

이 물질의 구성 성분은 다당류와 단백질이었으며 다당류는 한 종류의 미확인 물질을 포함한 7종의 단당으로 구성되어 있고 단백질은 14종의 아미노산으로 구성되어 있었다.

마우스에 sarcoma 180을 이식한 후 자실체의 단백질-다당류를 1일 20mg과 50mg/kg씩을 각각 투여했을 때 75%와 65%의 높은 저지율을 나타내었으며, 배양균사체에서 추출한 단백질-다당체는 1일 20mg/kg씩 투여시 58%의 항암효과를 나타내었다.

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