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Antitumor Components of *Ramaria formosa*

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韓國產 高等 菌類의 成分 硏究(제34보)

붉은싸리버섯의 抗癌 成分

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Abstract: To investigate antitumor components of Korean higher fungi, the carpophores of *Ramaria formosa* (Fr.)Quél. were collected in Gang Won Province and extracted with hot water. The extract was concentrated and precipitated by four volumes of ethanol. The precipitate was centrifugated and purified by dialyzing through Visking tube and a protein-bound polysaccharide fraction was obtained. The fraction was tested for antitumor activity against sarcoma 180 implanted in mice. The tumor inhibition ratio of the fraction was 66% in the dose of 50mg/kg/day for the period of ten days. The tumor in two of the eight mice was completely regressed. The components of the fraction were found to be a polysaccharide and a protein. The chemical analysis of the fraction showed that the polysaccharide moiety consisted of four monosaccharides and that the protein moiety contained fourteen amino acids.

Although more than 600 species of basidiomycetes have been recorded in Korea, investigations on their components had been scarce until our laboratory began to study on alkaloids(Kim *et al.*, 1970), fatty acids (Kim *et al.*, 1978; Lee *et al.*, 1979), amino acids (Kim *et al.*, 1977; Kim *et al.*, 1980; Yang *et al.*, 1980), sterols (Kim *et al.*, 1976; Kim *et al.*, 1976; Kim *et al.*, 1978; Kim *et al.*, 1978; Kim *et al.*, 1979) and antibacterial substances (Kim *et al.*, 1978). Recently it was reported that the protein-bound polysaccharides obtained from *Lentinus edodes, Pleurotus ostreatus, Coriolus versicolor* and *Ganoderma lucidum* exhibited high antitumor activities *in vivo* (Kim *et al.*, 1979; Park *et al.*, 1979; Min *et al.*, 1980; Nakanishi *et al.*, 1963; Nakahara *et al.*, 1964; Tanaka *et al.*, 1965; Tanaka, 1967; Shim, 1980; Nakahara *et al.*, 1967; Chihara *et al.*, 1969; Komatsu *et al.*, 1969; Ikekawa *et al.*, 1969; Sasaki *et al.*, 1967; Fukuda *et al.*, 1975; Shimura *et al.*, 1978; Fujii *et al.*, 1978; Suzuki *et al.*, 1978; Keller, 1977). These reports led us to search anticancer components with low toxicity in the basidiomyces of Korea.

For this purpose, *Ramaria formasa* (Fr.)Quél., one of the wild basidiomycetes which is used for food, was extracted with hot water, and the extract was tested for antitumor activity against sarcoma 180 implanted in mice. The chemical analysis of the extract showed that it contained a protein and a polysaccharide.

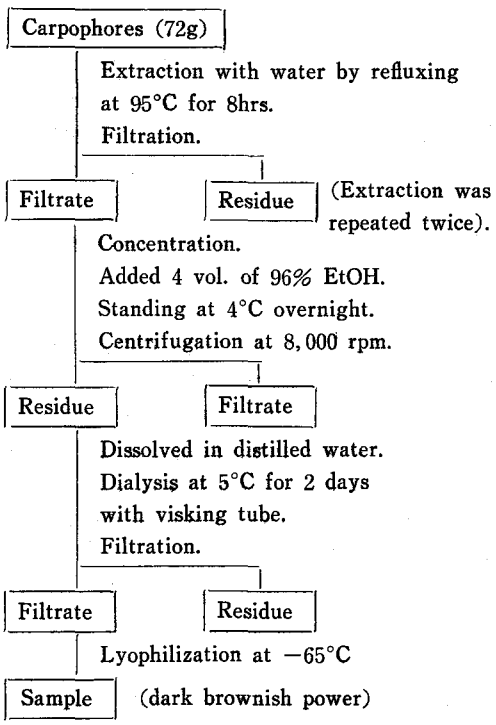
Materials and Methods

Fungal Material

The carpophores of *Ramaria formosa* used in this study were collected in Gang-Won Province during the period from July to August 1981.

Extraction and Isolation

Seventy two grams of the dried carpophores of *R. formosa* were homogenized in a Waring blender and extraction was carried out three times. Extraction was performed by stirring and refluxing at 95±5°C on a hot water bath for eight hours. After filtration, the residue was extracted with 800ml hot water and then 400ml hot water under the same condition for five hours, respectively. All the filtrates were combined to be concentrated in a rotary vecuum evaporator to 400ml (Scheme I).



Scheme I. Extraction and isolation of *R. formosa.*

The filtrate was mixed with four volumes of 96% ethanol and allowed to stand at 4°C overnight. The precipitate was collected by centrifugation for 30 minutes at 8,000 rpm at 20°C(Beckman model J-21 centrifuge) and redissolved for lyophilization. The lyophilized powder was then dissolved in 300ml of distilled water and dialyzed at 5°C for seven days with visking tube. After dialysis, the insoluble substance was removed by filtration. The filtrate was concentrated in a rotary vacuum evaporator and dried at −65°C in a lyophilizer (Edwards high vacuum model No. EFD3). An odorless and tasteless dark brownish powder was obtained. This powder was used in the following experiments.

Chemical Analysis of the Extracts

The concentration of test solutions containing the fraction of *R. formosa* was 1% (w/v) in all cases. Molish test, Anthrone test, Iodine test, Ninhydrin test, Ninhydrin test after acid hydrolysis, Lowry-Folin test, and Biuret test were conducted according to the previous methods (Park *et al.*, 1979).

Assay for Polysaccharide of the Extract

1) Polysaccharide content

Polysaccharide content of the extract was quantitatively calculated by Anthrone method using glucose as a standard sugar. After the extract and glucose were processed by the method, degree of absorption was measured by Hitachi Recording Spectrophotometer at 625nm. Polysaccharide content was calculated from the calibration curve.

2) Sugar analysis

Twenty milligrams of the extract were dissolved in 2ml of 3% hydrochloric acid-methanol in an ampule. The ampule was filled with nitrogen gas in order to prevent oxidation, and then sealed. Methanolysis was carried out at 100±5°C for 20 hours. The methanolysate was filtered to remove the precipitate. The filtrate was neutralized with 2N NaOH and dried at −65°C in a lyophilizer (Edward high vaccum model No. EFo3) for four hourr. After dissolving in one ml pyridine, trimethylsilylation was carried out using 0.2ml of hexamethyldisilazane and 0.1ml of trimethylchlorosilane. Gas liquid chromatography(Pye Unicam) was performed under the condition in Table I. Ten milligrams of each standard sugar were examined by G.L.C. by the same condition. Several monosaccharides of the extract were

identified by comparison with retention times of authentic standard sugars. The content of each monosaccharide was calculated from the chromatograms by HW law and planimetry.

Assay for Protein of the Extract

1) Protein content

Protein content of the extract was determined by Lowry-Folin method using albumin as a standard protein with Hitachi Recording Spectrophotometer at 750nm.

2) Amino acid analysis

Two hundred milligrams of the extract were dissolved in 50ml of 6N-HCl and divided into ampules. The ampules were filled with nitrogen gas in order to prevent oxidation and then sealed. Hydrolysis was carried out at 110±5°C for 20 hours. The hydrolysate was filtered to remove the precipitate and evaporated to dryness. The dried substance was dissolved in 10ml of 0.1N HCl and diluted with 0.2M sodium citrate buffer (pH 2.2). Under the usual conditions (Kim *et al.*, 1980), amino acids were analyzed with Hitachi amino acid auto analyzer Model KLA-5. Standard amino acids were also analyzed under the same condition and a chromatogram was also obtained. The amino acid mixture used for standardization contained 0.1μmol of each amino acid (in case of proline, 0.2μmol) dissolved in 0.5ml of 0.2M sodium citrate buffer (pH 2.2). Contents of each amino acid were calculated from the chromatograms by HW law.

Antitumor Test

1) Tumor cell

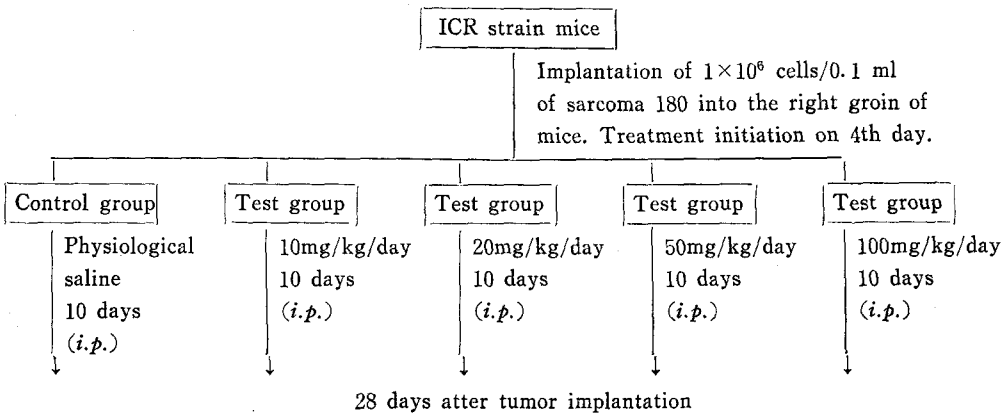
ICR-strain mice of male sex weighing about 20~25g were used in this experiment. Sarcoma 180 cells were implanted into the intraperitoneal cavity of the mice. After 10 days of implantation, the animals were killed and sarcoma 180 cells were collected with a syringe in an ice-cold bath. This ascitic fluid was diluted with saline to adujust the tumor cell concentration to 1×10^7 cell/ml.

2) Preparation of test solution

To prepare a test solution for a dose of 10mg/kg, 50mg of the extract powder which was obtained from the carpophores of *R. formosa* were dissolved in 20ml of saline. And 100mg, 250mg, 500mg of the powder were dissolved in 20ml of saline and solutions for dose of 20mg/kg, 50mg/kg, 100mg/kg were prepared. For control, physiological saline was used. These solutions were autoclaved for 20 minutes and stored in a refrigerator.

3) Animal test

To test the extract for antitumor effect as shown in Scheme II, five groups of 10 mice (ICR mice of male sex weighing about 20~25g) were respectively inoculated with 0.1ml of ascitic tumors (1×10^6 cell/0.1ml) into the right groin of the mouse. The administration of the test solution was initiated on the fourth day after tumor implantation and continued daily for 10 consecutive days. To the first group of 10 mice, 0.1 ml of solution at a dose of 10mg/kg



Scheme II. Antitumor test of the extract of *R. formosa* on mice bearing sarcoma 180.

was injected intraperitoneally. The second group was injected with 0.1 ml of the solution at a dose of 20 mg/kg. The third group was injected with 0.1 ml of the solution at a dose of 50mg/kg.

The fourth group was injected with 0.1 ml of the solution at a dose of 100mg/kg. The last group was injected intraperitoneally with 0.1 ml of physiological saline and used as control group. Tumor weights were measured on the 28th day after implantation and inhibition ratio (I.R.) was determined by comparison with tumor weights of the control group. Tumor inhibition ratio was calculated as follows:

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

Tw=Average tumor weight of each treated group.

Cw=Average tumor weight of the control group.

Results

Yield of the Extract

From 72g of the dried carpophores of *R. formosa*, 2.7g of the aqueous extract was obtained (yield 3.75%).

Chemical Analysis of the Extract

The results in Table I showed that the extract consisted of a protein and a polysaccharide.

Assay for Polysaccharide of the Extract

1) Polysaccharide content

The polysaccharide in the extract was 34.0%.

2) Analysis of monosaccharides of the polysaccharide moiety.

Table I. Results of various color reactions on the extract of *R. formosa.*

Method	Result
Molish test	purple
Anthrone test	dark green
Iodine test	negative
Ninhydrine test	blue violet
Ninhydrine test after acid hydrolysis	violet
Lowry-Folin test	dark blue
Biuret test	purple blue

The monosaccharides of the polysaccharide moiety of the extract were found to be glucose, fucose, galactose and xylose as shown in Table II. And their ratio was also shown in Table II. G.L.C. pattern of the monosaccharides of the moiety was shown in Fig. 1.

Assay for Protein of the Extract

Table II. The contents of the polysaccharide and the monosaccharides of the extract of *R. formosa.*

Polysaccharide content(%)*	34.0%
Monosaccharide content (%)**	
Glucose	25.3
Fucose	5.3
Galactose	54.4
Xylose	5.0

* Percentage to the aqueous extract.

** Percentage to the polysaccharide moiety of the extract.

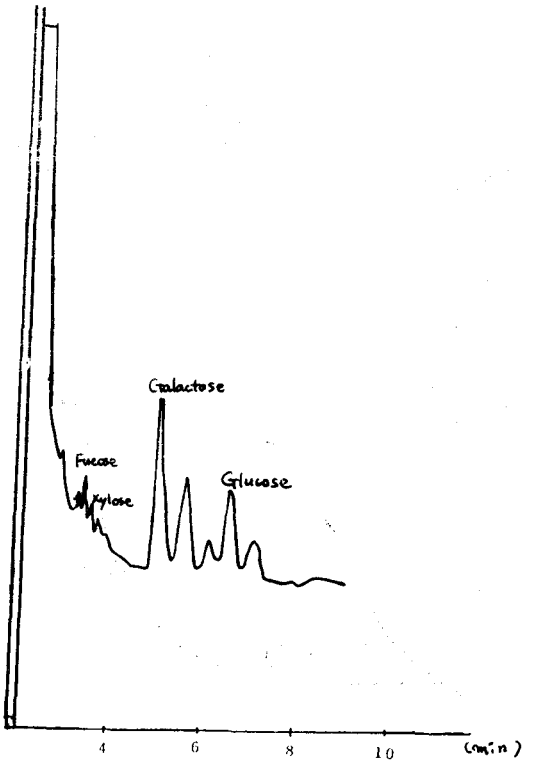


Fig. 1. GLC pattern of the monosaccharides of the extract of *R. formosa.*

The results of Lowry-Folin test showed that the protein content of the extract was 40.2% (Table IV). The percent of each amino acid of the protein moiety was shown in Table V. The chromatogram of the amino acids of the protein moiety of the extract was shown in Fig. 2. The protein moiety was composed of 14 amino acids.

Table III. Retention time of standard TMS-monosaccharides by G.L.C.

Monosaccharide	Retention time
Fucose	2.8 min
Xylose	3.2
Mannose	7.0
Fructose	7.8
Galactose	7.4
Glucose	9.6

Table IV. Contents of protein of the antitumor fraction of *R. formosa.*

Total contents (%) of protein after Lowry-Folin test at 750nm	40.2%

Table V. Contents of amino acids of the protein moiety of the antitumor fraction of *R. formosa.*

Amino Acid	Content (%)*
Lysine	21.6
Arginine	3.2
Aspartic acid	13.6
Threonine	8.6
Serine	9.2
Glutamic acid	8.2
Cysteine	3.9
Methionine	2.9
Alanine	10.4
Isoleucine	4.4
Leucine	1.1
Tyrosine	2.9
Glycine	10.0
Proline	trace

* Percentage to the protein moiety.

Antitumor Test

Antitumor effects of the extract of *R. formosa* on sarcoma 180 in mice were shown in Table VI. The

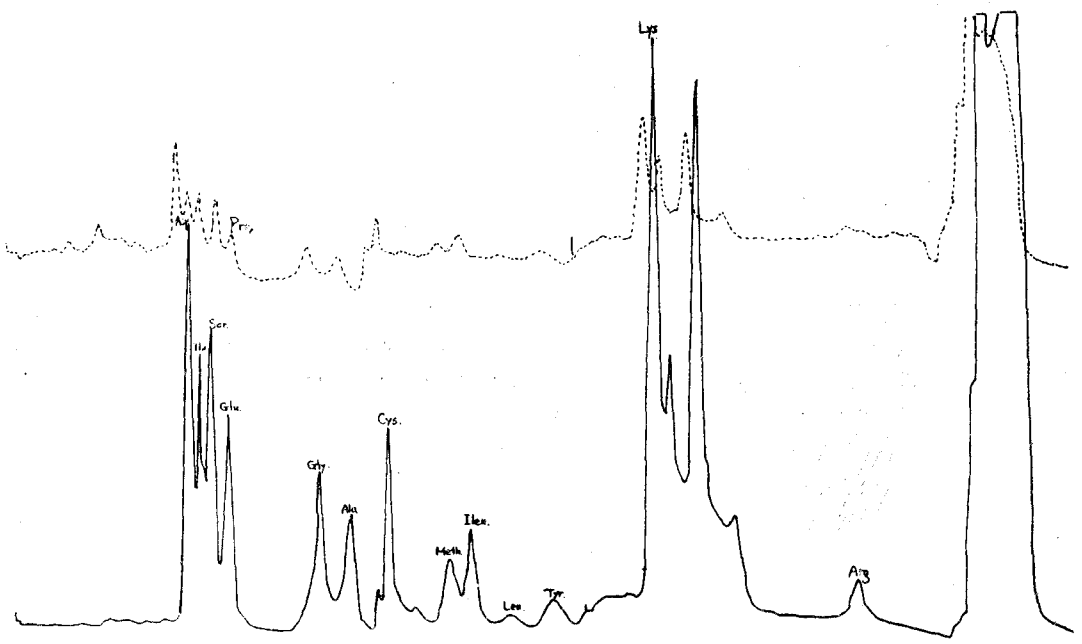


Fig. 2. Chromatogram of amino acids of the protein moiety of the antitumor fraction of *R. formosa.*

Table VI. Antitumor effects of the protein-bound polysaccharide of *R. formosa* on mice bearing sarcoma 180.

	Average tumor weight(g)	Inhibition ratio (%)	100% Regression
Control	6.36±2.83	—	—
10mg/kg	4.59±1.31	28%	0/8
20mg/kg	5.00±2.47	21%	0/8
50mg/kg	2.17±2.00	66%	2/8
100mg/kg	2.36±1.67	63%	2/8

* Values are means ± standard deviation.

effects of the therapeutic administration of the extract on the life span of the mice with sarcoma 180 was shown in Fig. 3. From this Figure, the life span of the treated groups was longer than of the control group. In the therapeutic effects on sarcoma 180 in the mice, 50mg/kg i.p. administration of the extract was most effective on the inhibition of tumor growth.

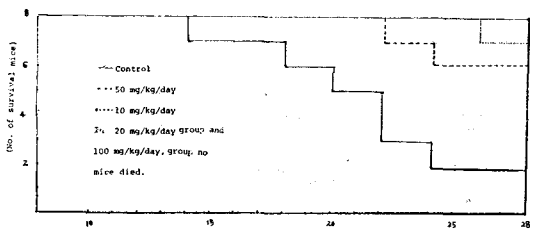


Fig. 3. Therapeutic effect of the extract on the life span of the mice inoculated with sarcoma 180.

Discussion

The protein-bound polysaccharide of *Ramaria formosa* showed an antitumor activity against sarcoma 180 in mice. The dose of 50 mg/kg/day exhibited the highest activity, but it seemed to be less active than those of other basidiomycetes (Kim *et al.*, 1979; Min *et al.*, 1980; Lee *et al.*, 1981; Kim *et al.*, 1982), although they are not directly comparable with one another.

The chemical analysis of the antitumor fraction showed that it contained a protein (40%) and a polysaccharide (34%) and that the protein moiety was the major component. The latter fact is unusual since the polysaccharide was the major component in the antitumor fractions of other basidiomycetes, except *Naematoloma fasciculare* (Lee *et al.*, 1981) and *Ganoderma lucidum* (Kim *et al.*, 1980). The polysaccharide moiety of the antitumor fraction may be unique, since galactose was the major subunit in it. It was found that glucose was the major monosaccharide in those of other antitumor fractions, except *Naematoloma fasciculare* and *Cryptoporus volvatus* (Kim *et al.*, 1982). It was of interest that the protein moiety contained a large amount of lysine (21.6%), whereas glutamic acid (*C. volvatus*, *N. fasciculare*, and *P. ostreatus*), aspartic acid (*S. commune*, *A. auricula-judae*, *L. edodes*, and *C. versicolor*), and cysteine (*G. lucidum*) were the major amino acid respectively. The chemical composition of this antitumor protein-bound polysaccharide appears to be distinctively different from those of the antitumor components of other basidiomycetes of Korea that we have so far investigated. Hence further studies on its chemical structure and immunological properties are warranted.

Conclusion

The protein-bound polysaccharide of *R. formosa* of Korea showed an antitumor activity against sarcoma 180 implanted in mice. The inhibition ratio of the tumor was 66%. The antitumor fraction was found to be a protein-bound polysaccharide. The polysaccharide moiety consisted of glucose, fucose, galactose and xylose, and the protein moiety contained 14 amino acids.

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요 약

한국산 고등 균류의 항암성분을 연구하고자 강원도에서 채집한 붉은싸리버섯 *Ramaria formosa* (Fr.)Quél. 로 부터 추출, 농축, 침전시킨후, 그 침전을 원심분리하고 Visking tube를 사용하여 정제한 결과 담백질-다당체를 얻었다. 이 물질의 항암 효과를 쥐에 이식된 sarcoma 180에 대하여 시험하였으며 50 mg/kg/day의 용량으로 10일간 투여한 결과 그 종양억제율은 66%였다. 8마리중 2마리에서는 완전한 종양 퇴화를 관찰하였다.

이 항암 성분을 화학적으로 분석하여 그 다당체의 대부분은 4종의 단당으로 구성되었고 단백질 부분은 14종의 아미노산으로 구성되어 있음을 밝혔다.

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