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Studies on Constituents of Higher Fungi of Korea(XXXIII)

Antitumor Components of *Trametes sanguinea*

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韓國産 高等 菌類의 成分 硏究(제33보)

간버섯의 抗癌 成分

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Abstract: To find antitumor components with low toxicity from natural resources, antitumor test of the water extract of the carpophores of *Trametes sanguinea* (L. ex Fr.)Lloyd. was undertaken. The carpophores of this fungus were collected in Gyeong Gi Province and extracted with hot water. The extract was 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 against the tumor was 72.4% at the dose of 10mg/kg/day for the period of ten days. The tumor in one of the eight mice was completely regressed. The antitumor components were found to be a polysaccharide and a protein. The hydrolysis of the polysaccharide moiety yielded three monosaccharides, and from the hydrolysate of the protein moiety 13 amino acids were identified.

Although more than 600 species of Korean higher fungi have been identified up to date (Kim, 1978), there have not been so many reports on biologically active components of these fungi. Research reports on analyses of components of Korean higher fungi have been made mainly by our laboratory (Kim *et al.*, 1970~1982). The components of the fungi, such as alkaloids, fatty acids, amino acids, sterols and antimicrobial substances were reported by us (Kim *et al.*, 1970~1978). Recently it has been reported that the aqueous extracts from several of Basidiomycetes such as, *Coriolus versicolor*, *Pleurotus ostreatus*, *Lentinus edodes*, *Ganoderma lucidum*, *Schizophyllum commune* and *Auricularia auriculajudae* had very high antitumor activity against sarcoma-180 implatned in mice (Chihara *et al.*, 1969; Fukuda *et al.*, 1975; Ikekawa *et al.*, 1969; Kim *et al.*, 1979; Kim *et al.*, 1980b; Kim *et al.*, 1980c; Kim *et al.*, 1981; Komatsu *et al.*, 1969; Nakahara *et al.*, 1964 and 1967). To find out antitumor components with low toxicity from natural products of Basidiomycetes, the carpophores of *Trametes sanguinea*, a wild mushroom in Korea were extracted with hot water, and its effect on the growth of sarcoma-180 implanted in mice was examined. Here this paper reports the extraction, and purification procedure and chemical analysis of the protein-bound polysaccharide of *T. sanguinea* and its antitumor effects on tumor bearing animals.

Materials and Methods

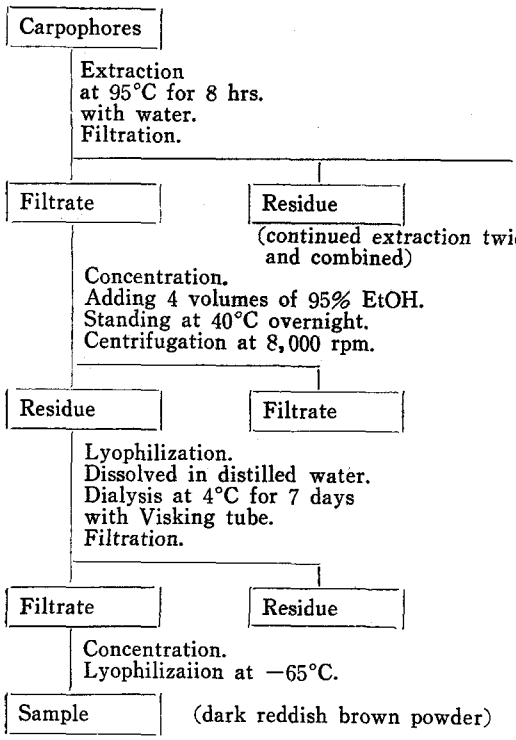
Fungal Material

The carpophores of the fungus, *Trametes sanguinea*

(L. ex Fr.) Lloyd, used in this study were collected at Su-won in Gyeong-Gi Province during the period from May 1979 to August 1980. It belongs to the family *Polyporaceae* and is one of the wood-rotting mushrooms (Imazeki and Hongo, 1957).

Extraction and Isolation

Thirty grams of the dried carpophores of *T. sanguinea* were homogenized with 1000ml of hot water for five minutes in a Waring blendor. Extraction was performed by stirring and refluxing on a water bath of 95±5°C for eight hours. After filtration, the residue was extracted under the same condition with 800ml and then 400ml of the same solvent for eight hours, respectively. All the consequent filtrates were combined and then concentrated to 800ml in a rotary vacuum evaporator. The concentrated filtrate was mixed with four volumes of 95% ethanol and allowed to stand at 4°C overnight. The precipitates were collected by centrifugation for 30 minutes at 8,000 rpm at 20°C (Beckman model J-21 centrifuge Rotar JA-14) and redissolved for lyophilization. The lyophilized powder was then dissolved in 300ml of distilled water and dialyzed at 4°C for seven days using Visking tube by changing the distilled water. After dialysis, insoluble substances were 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. EF03). An odorless and tasteless reddish brown powder was obtained (Scheme I). This powder was used as the protein-bound polysaccharide fraction in the following experiments.



Scheme I. Extraction and isolation of aqueous extract of *T. sanguinea*.

Antitumor test

1) Tumor cells

Sarcoma 180 cells were implanted into intraperitoneal cavity of ICR mice of male sex weighing about 20g. After incubation for ten days, the animals were killed and sarcoma 180 ascitic fluid was collected with a syringe in a beaker immersed in an ice bath. After washed twice or thrice with saline, cell suspension was diluted to adjust the tumor cell concen tration to 1×10^7 cell/ml.

2) Preparation of the test sample solution

To prepare a solution for a dose of 10mg/kg, 20mg of the reddish brown powder which was obtained from the carpophores of *T. sanguinea* were dissolved in 10 ml of saline. Also 100mg of the powder were dissolved in 10ml of saline for a dose of 50mg/kg. For control, saline was used. These solutions were autoclaved at 121°C for 20 minutes and stored in a refrigerator.

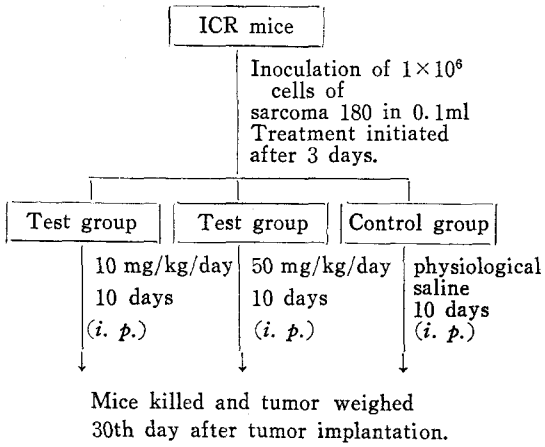
3) Antitumor test *in vivo*

Experiments of antitumor activity of the extract were conducted by the method shown in Scheme II. Three groups of 8 mice (ICR mice of male sex weighing about 20g) were respectively inoculated with 0.1ml of ascitic tumors (1×10^7 cell/ml) into the right groin. The administration of the sample solution was initiated on the third day after tumor implantation and continued once daily for 10 consecutive days. The low-dose group of 8 mice was intraperitoneally injected with 0.1 ml of the solution at a dose of 10 mg/kg. The high-dose treated group was injected with 0.1 ml of the solution at a dose of 50 mg/kg. The control group

was injected intraperitoneally with 0.1 ml of saline. Tumor weights were measured on the 30th day after implantation. Tumor inhibition ratio 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.



Scheme II. Antitumor test of the extract of *T. sanguinea* on sarcoma 180 in mice.

Chemical Analysis of Extract

The concentration of test solutions containing the extract of *Trametes sanguinea* used in the reactions was 1% (w/v) in all cases.

1) Anthrone test:

Two milliliters of anthrone reagent (0.2% conc. sulfuric acid solution) were added to two milliliter test solution and mixed completely.

2) Molish test:

Two drops of α-naphthol reagent (5.0% ethanol solution) were added to two milliliter test solution. After shaking, one milliter of conc. sulfuric acid was carefully poured.

3) Iodine test:

One drop of dilute hydrochloric acid was added to two milliliters of test solution and two drops of iodine solution were added.

4) Xanthoprotein test:

One milliter of conc. nitric acid was added to one milliter of test solution.

5) Trytophane test:

Seven milliliters of 77% sulfuric acid were added to one milliliter of test solution and cooled to 10~15° with water. One milliliter of fresh one percent tryptophane solution was added to the above solution and heated on a boiling water bath for 20 minutes and cooled to room temperature.

6) Biuret test.

Three drops of cupric sulfate solution were added to two milliliters of test solution. After two milliliters of 10% sodium hydroxide solution were added and mixed thoroughly, the color change was observed.

7) Ninhydrin test:

Two milliliters of 1% ninhydrin solution were added to two milliliters of test solution (neutral pH) and heated on a boiling bath for two minutes.

8) Ninhydrin test on hydrolysate:

The sample was hydrolyzed in 6N-hydrochloric acid at 110° for 24 hours in an ampule filled with nitrogen gas. After filtration ninhydrin test was conducted.

9) Lowry-Folin test: One hundred milliliters of fresh alkaline copper reagent were prepared in order of one ml of 1% cuppuric sulfate, one ml of 2% sodium tartrate and 98ml of 2% sodium carbonate in 0.1N-sodium hydroxide solution. Three solutions were thoroughly mixed in a 100ml Erlenmyer flask immediately. Five milliliters of alkaline copper reagent were added to one milliliter test solution. After ten minutes, 0.5ml of Folin-Ciocalteu reagent was added and mixed thoroughly. After 30 minutes the color change was observed.

Assay for Polysaccharide of the Extract

1) Total 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 U.V. spectrophotometer (Unicam SP 1805 Programme Controller) at 625 nm. Polysaccharide content was calculated from the

calibration curve.

2) Sugar analysis: Twenty milligrams of each standard sugar were respectively dissolved in 2 ml of 3% hydrochloric acid-methanol in an ampule. It was filled with nitrogen gas in order to prevent oxidation and sealed. Methanolysis was carried out at 100± 5°C for 20 hours. The methanolysate was filtered to remove the precipitae and evaporated. After dissolving in one ml of 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. 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.

Table I. Measurement condition of GLC.

Column	3% OV-17 (80-100 mesh shimalite) 3mm ID×1m boronsilicate glass column
Temperature	Column: 160°C Detector: 190°C
Flow rate	N_2: 50ml/min H_2: 60ml/min (0.8kg/cm²) A_{ir}: 88ml/min (1.2kg/cm²)
Attenuation	8×10² a.f.s. (amperé full scale)

Assay for Protein of the Extract

1) Total protein content

Protein content of the extract was determined by Lowry-Folin method using albumin as a standard protein with U.V. spectrophotometer (Unicam SP 1805 Programme Controller) at 750nm.

2) Amino acid analysis

Twenty milligrams of the extract were dissolved in 5ml of 6N hydrochloric acid and poured 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. The hydrolysate was filtered to remove the precipitate and evaporated to dryness. The dry substance was dissolved in 2ml of 0.02N-hydrochloric acid solution. Under the condition of Table II, amino acids were analyzed by an amino acid auto analyzer (Hitachi Model 835). Standard amino acids were also analyzed under the same condition and a chromatogram was also obtained. The amino acid mixture used for standardization contained 3 nmol/40μl of each amino acid. Contents of each amino acid were calculated from chromatograms by peak height method.

Table II. Measurement conditions of amino acid analyzer.

Column	2.6×150mm
Ion exchange resin	#2619 (Hitachi)
Flow rate	Buffer soln 0.225 ml/min ninhydrin 0.3ml/min
Analysis cycle time	70 min
Column pressure	80 130kg/cm²
Ninhydrin pressure	15 35kg/cm²
Column temp.	53°C
N_2 gas pressure	0.28kg/cm²
Reaction bath temp.	98°C
Wave length	570nm, 440nm

Results

Yield of the protein-bound polysaccharide fraction

From 30g of the dried carpophores of *T. sanguinea*, 2.4g of the fraction was obtained (yield 8.0%).

Antitumor test

Antitumor activity of the fraction from *T. sanguinea* on sarcoma 180 in mice was shown in Table III. Complete regression was also examined on the thirtieth day after the tumor implantation. The tumor in two of the 8 mice of 10 mg/kg/day group was completely regressed. The effects of the fraction on the life span of mice with sarcoma 180 were shown in Fig. 1. Also the tumor of one of the 8 mice of 50mg/kg/day group was completely regressed. The prolonged life span of the treated mice also showed the antitumor activity of the sample.

Chemical assay for the fraction

The results shown in Table IV suggest that the extract consist of a protein and a polysaccharide.

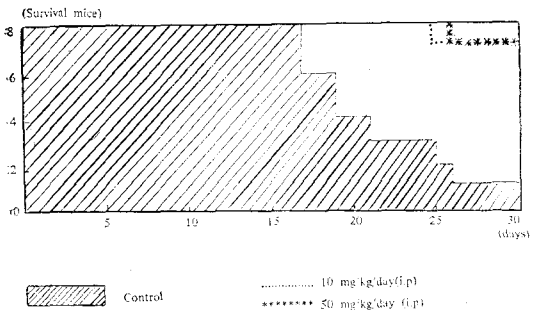


Fig. 1. Effects of *Trametes sanguinea* extract on the life span of mice inoculated with sarcoma 180 intaperitoneally.

Table III. Antitumor effects of the protein-ouund polysaccharide of *T. sanguinea* on sarcoma 180 in mice.

	Average tumor weight(g)	Inhibition ratio (%)	100% regression
Control (8 mice/group) *i.p.* saline	9.26±0.81*	—	—
10 mg/kg/day (*i.p.*)	2.56±0.42**	72.4	2
50 mg/kg/day (*i.p.*)	2.73±0.28**	70.5	1

* Values are means ± standard deviation
** ($P<0.01$)

Table IV. Results of various color reactions on the extract from *T. sanguinea*

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

Assay for polysaccharide of the fraction

1) Polysacchide content

The polysaccharide in the fraction was 55.6%.

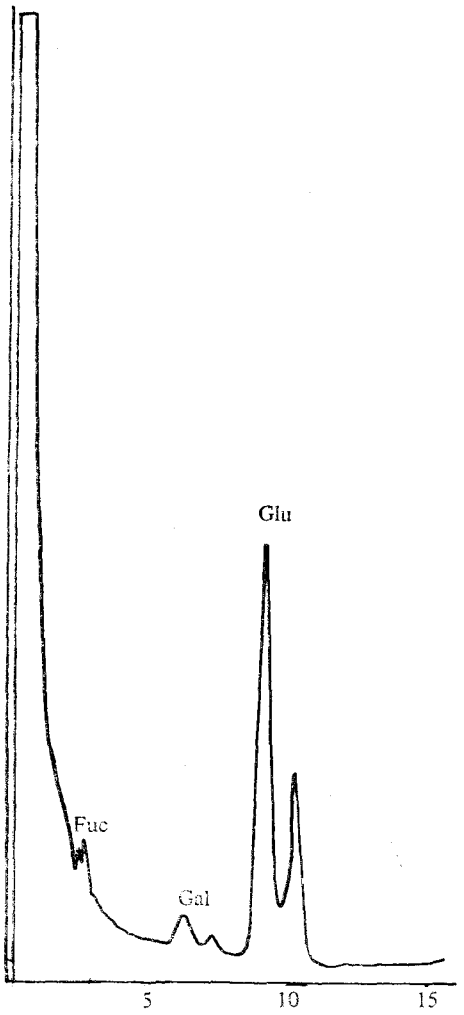


Fig. 2. G.L.C. pattern of the monosaccharides of the polysaccharide moiety of the antitumor fraction of *T. sanguinea.*

2) Analysis of the monosaccharides of the polysaccharide moiety

The monosaccharides of the moiety were found to be fucose, galactose and glucose as shown in Table V. And their percentages were also shown in Table V. The GLC pattern of the monosaccharides of the extract was shown in Fig. 2. Table VI, shows the retention times of authentic sugars.

Analysis of the protein moiety of the fraction and the contents of amino acids in the protein moiety

The results of Lowry-Folin test showed that

Table V. The polysaccharide and the monosaccharide contents of the polysaccharide moiety of the antitumor fraction of *T. sanguinea.*

	Content (%)
Total content (%) (after Anthrone test at 625 nm)	55.6
Monosaccharide content (%)	
Fucose	4.3
Glucose	88.0
Galactose	7.7

Table VI. Retention times of the TMS-monosaccharides by GLC.

TMS-monosaccharide	Retention time
Xylose	3.4, 3.6
Fucose	2.4
Galactose	6.4, 7.4
Glucose	9.0, 10.1

the protein content of the fraction was 27.0%. The ratio of the amino acids of the protein moiety was shown in Table VII. The chromatogram of amino acids of the protein moiety was shown in Fig. 3. Although standard amino acids were analyzed

Table VI. Protein and amino acids contents in the protein moiety of the antitumor fraction of *T. sanguinea.*

	Content (%)
Total content (%) (after Lowry-Folin test, measured at 750nm)	27.0
Amino acid content (%) (detected by a.a. analyzer)	
Aspartic acid	11.53
Threonine	7.56
Serine	9.48
Glutamic acid	9.95
Glycine	12.89
Alanine	18.95
Cysteine	7.26
Methionine	4.30
Tyrosine	2.84
Lysine	5.72
Arginine	4.23
Proline	trace
Isoleucine	5.50

in the same manner and their chromatogram was obtained, it was not shown here. The protein moiety was composed of 13 amino acids which included

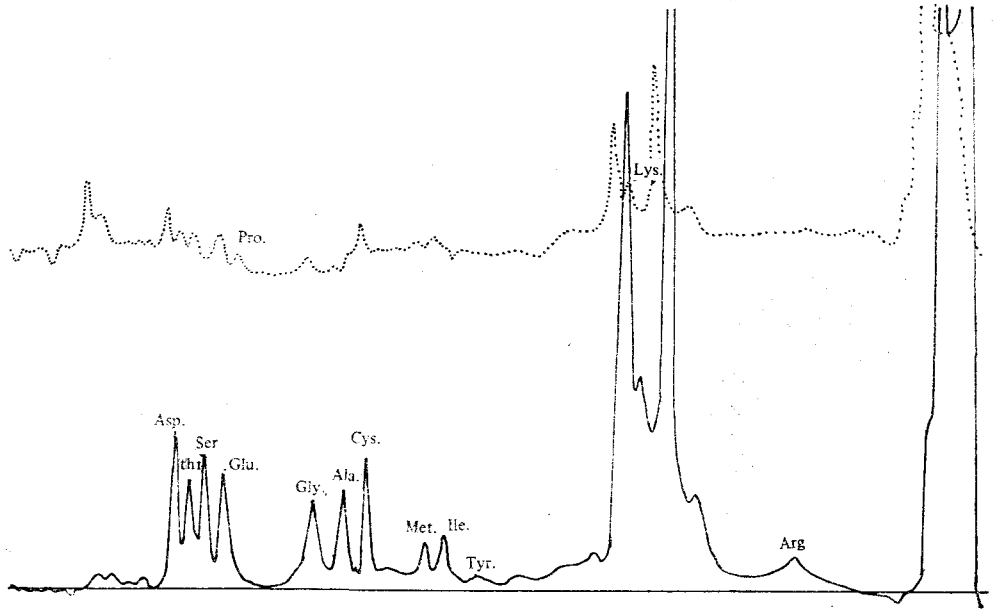


Fig. 3. Chromatogram of amino acids of the protein moiety of the antitumor fraction of *Trametes sanguinea.*

aspartic acid, glycine and alanine as major amino acids.

Discussion

The results showed that the protein-bound polysaccharide fraction of *Trametes sanguinea* had a relatively high antitumor activty in the animal test. That is, the fraction showed 72.4% tumor inhibition ratio against sarcoma 180 implanted in mice at a dose of 10 mg/kg/day. And 70.5% tumor inhibition ratio was observed at a dose of 50 mg/kg/day. The results indicate that the response was not dose-dependent. The mechanism of the antitumor action appears to be indirect rather than direct. It was recently proposed that the antitumor activity of the polysaccharides of other high fungi may be due to activated macrophages and lymphocytes. In other words, the antitumor fraction might be an immunopotentiator, but not cytotoxic agent. The carpophores of *T. sanguinea* are not abundunt in natural habitat. So it is desirable to isolate the mycelia of this fungs, cultivate them in liquid media, and extract the antitumor fraction from the mycelia. The content of the polysaccharide was slightly greater than those of the polysaccharides of other fungi and the major monosaccharide was glucose. The data strongly suggest that the polysaccharide moiety may be different from those of other antitumor fractions.

In summary, the carpophores of *Trametes sanguinea* contained a unique immunopotentiating antitumor protoin-bound polysaccharide.

Conclusion

The protein-bound polysaccharide fraction of *Trametes sanguinea* of Korea showed a high antitumor activity against sarcoma 180 in mice. The antitumor extract was found to be a polysaccharide and a protein. The polysaccharide moiety consisted of glucose, fucose and galactose, and the protein moiety contained 13 amino acids, including glutamic acid and aspartic acid.

요 약

한국산 고등균류의 성분중에서 항암성분을 탐색하고자, 경기도 수원에서 채집한 갈버섯 *Trametes sanguinea* (L. ex Fr.) Lloyd.로 부터 무미 무취인 짙은 갈색 분말을 추출 분리하였다. 이 물질은 마우스에 이식된 sarcoma 180에 대해 높은 저지율을 보였고, 그 저지작용은 이 물질의 치료적인 투여에 의해서 나타났다. 이 물질의 성분은 다당류와 단백질이었으며, 다당류는 fucose, glucose와 galactose의 3종의 당을 함유하였다. 단백질 분획은 13종의 아미노산으로 구성되었으며, 특히 산성 아미노산이 많은 반면에 염기성 아미노산은 적었다.

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