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Antitumor Components of *Cryptoporus volvatus*

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한입버섯의 抗癌成分에 관한 硏究

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Abstract: The carpophores of *Cryptoporus volvatus* collected in Gyeong-gi Province of Korea were extracted with water and a protein-polysaccharide fraction was obtained after dialysis and lyophilization. The antitumor activity of this fraction was tested against sarcoma 180 implanted in A-strain mice. The tumor inhibition ratio was 80.4% in case of the high dose group (50mg/kg, ip, 10 days) and 70.3% in the low dose group (20mg/kg, ip, 10 days). The protein-polysaccharide fraction was chemically analyzed and was found to be a complex of a protein which was 18.2% of the fraction when determined by Lowry-Folin method, and a polysaccharide which was 55.3% of ther fraction when determined by Anthrone method. Their subunits were identified as four monosaccharides and 18 amino acids by gas-liquid chromatography and amino acid autoanalysis.

It was reported that BCG (Keder *et al.*, 1973; Masuno *et al.*, 1979; Chikama *et al.*, 1979) and some other bacterial adjuvants such as *Corynebacterium parvum*(Scott, 1974; Rivers-Moreno *et al.*, 1974; Peters *et al.*, 1977; Gebhardt and Fisher, 1979; Goldmann and Bar-Shavit, 1979), *Mycobactrium smegmatis* (Lamensans *et al.*, 1975; Juy and Chedid, 1975), and *Streptococcus haemolyticus*(Mashiba *et al.*, 1979; Torikai *et al.*, 1980), activate host response against tumor growth. Local application of these bacterial adjuvants causes regression of established tumors and the systematic administration can induce systematic resistance to syngeneic tumor transplants.

Recently it was reported that polysaccharides from certain higher fungi, for example, lentinan from *Lentinus edodes*(Chihara *et al.*, 1969; Maeda and Chihara, 1971; Park *et al.*, 1979; Chung, 1982), and PS-K from *Coriolus versicolor*(Ohno *et al.*, 1975; Namoto *et al.*, 1975; Ohno *et al.*, 1976; Usui *et al.*, 1976; Kim *et al.*, 1979; Park *et al.*, 1979; Shim, 1981), also have high antitumor activities against sarcoma 180 and other tumors. When compared with BCG and other bacterial adjuvants, these polysaccharides of basidiomycetes are superior to the former in that they are almost nontoxic.

There is a possibility to use these immunopotentiators of basidiomycetes as an immunotherapeutic agent in cancer therapy without causing acute toxicity.

Considering many species of higher fungi which await surveys on their constituents with antitumor activities, we endeaver to find other potent immunopotentiaters besides those previously reported.

We have already reported on the antitumor activity

and chemistry of antitumor polysaccharides from some Korean higher fungi(Kim *et al.*, 1679; Park, *et al.*, 1979; Kim *et al.*, 1980; Min *et al.*, 1980; Kang *et al.*, 1981; Shim, 1982; Shim, 1982; Chung, 1982). This paper reports the result of the antitumor test with another species of Korean basidiomycetes.

Materials and Methods

1. Fungal Material

The carpophores of *Crytoporus volvatus*(Pk.) Hubb. (Polyporaceae) were collected at the Gwang-neung area in Gyeong-gi Province during the period from May, 1979 to July, 1980 (Fig. 1). The specimens were verified by Dr. Ji Yul Lee, Professor of Mycology, Seoul Women's College, Seoul, Korea. The collected carpophores were dried in open shade and kept in a glass bottle until used. Some of them are kept as a voucher specimen at the Department of Microbial Chemistry, College of Pharmacy, Seoul National University.

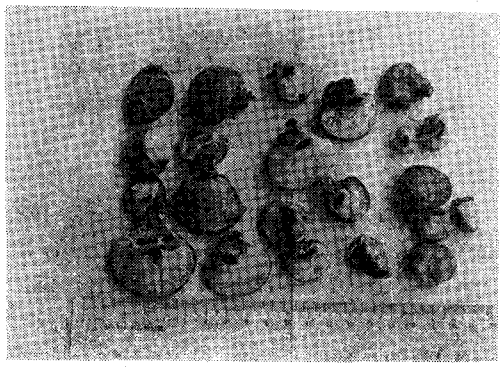


Fig. 1. Carpophores of *Cryptoporus volvatus*.

2. Extraction and Isolation

One hundred grams of the dried carpophores of *C. volvatus* were soaked with water for one hour at room temperature and then homogenized for five minutes in a Waring blender. Extraction was performed in a round-bottom flask by refluxing for eight hours on a boiling water bath, using two liters of distilled water as extracting solvent(Scheme I).

After filtration, the residue was extracted under the same condition with 600 ml and then 500 ml of distilled water for eight hours, respectively. All the filtrates were combined and condensed in a rotary vacuum evaporator to 500 ml. The condensed filtrate was dialyzed at 5° for seven days in a visking tube. After dialysis this extract was condensed into 350 ml and centrifuged for 40 minutes at 6,000×g in a centrifuge(Beckman model J-21) to precipitate the insoluble fraction. The supernatant was lyophilized in a freeze-dryer(Edwards high vaccum model No. EFO3) to yield 9.3g of dark brownish powder. This powder was used as the protein-polysaccharide fraction in the following experiments.

3. Antitumor Test

Animal and Tumor: A-strain mice of female sex weighing about 20 g were used. They were obtained from the Experimental Animal Farm of Seoul National University. Sarcoma 180 cells were maintained by serial passage into the mice by intraperitoneal inoculation. Ascitic cells were collected ten days after tumor graft by washing out the peritoneal cavity with ice-cold saline under aseptic condition. The cells were sedimentated by centrifugation at 200×g for five minutes, resuspended in saline and washed again. These tumor cells were diluted with saline to adjust the tumor cell concentration at 1×10^7 cells/ml.

Preparation of Test Solution: Two hundred milligrams of the protein-polysaccharide fraction were dissolved in 20 ml of saline to make a high concentration sample solution and 80 mg in the same volume of saline to make a low concentration solution. These solutions were autoclaved at 121° for 15 minutes and stored in a refrigerator. Before injection, the sample solutions were warmed on a water bath to dissolve the gel-like precipitate.

Animal Test: For tumor implantation, 0.1 ml of tumor cell suspension(1×10^7 cells/ml) was injected subcutaneously into the left armpit. The mice were divided into three groups of eight members each. Being initiated on the third day after tumor implantation, the injection of the sample solutions was continued for ten days. In the high does group, 50mg/kg of the fraction was injected once everyday, 20mg/kg in the low dose group and saline in the control group. Injection volume was 0.1ml in each case,

Average tumor weight was estimated on the 30th day after tumor transplantation and inhibition ratio was determined by comparing with the average tumor weight of the control group. Complete regression was also examined(Scheme II).

4. Assay for Polysaccharide of the Antitumor Fraction

Total Polysaccharide Content: Total polysaccharide content of the fraction was determined by the anthrone method using glucose as a standard sugar with Pye-Unicam UV Spectrophotometer at 625 nm.

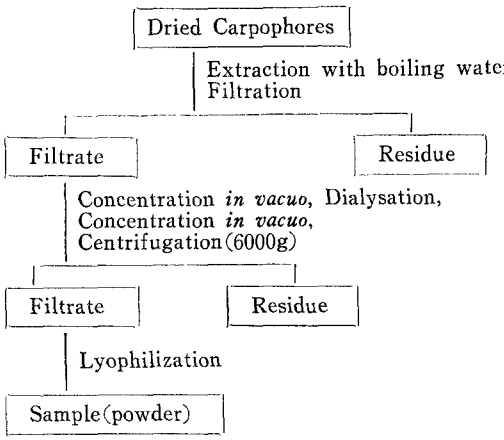
Determination of Monosaccharides: To determine subunits of the polysaccharide, 5mg of the antitumor fraction was dissolved in 3% hydrochloric acid-methanol(2ml) in an ampoule. The ampoule was filled with nitrogen gas and sealed. Methanolysis was carried out at $100 \pm 5°$ for 20 hours. The methanolysate was filtered and evaporated to dryness in a rotary vacuum evaporator. After the residue was dissolved in pyridine(1 ml), trimethylsilylation was carried out with 0.2 ml of hexamethyldisilazane and 0.1 ml of trimethylchlorosilane. Gas liquid chromatography was carried out under the conditions of the previous report(Park *et al.*, 1979), using authentic monosaccharides as standards. The content of each monosaccharide was calculated from the chromatogram by cutting and weighing the peak area.

5. Assay for Protein of the Antitumor Fraction

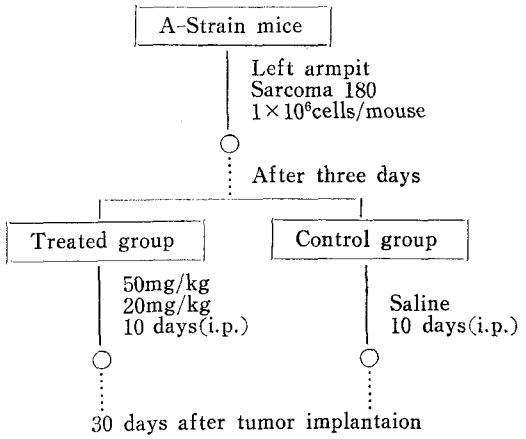
Total Protein Content: Total protein content of the fraction was determined by the Lowry-Folin method using egg albumin as a standard protein with Pye-Unicam UV Spectrophotometer at 750 nm.

Determination of Amino Acids: To determine subunits of the protein, 200 mg of the fraction was dissolved in 50 ml of 6N-hydrochloric acid and divided into 13 ampoules. The ampoules were filled with nitrogen gas, sealed and hydrolysed. Hydrolysate was filtered and evaporated in a rotary vacuum evaporator after hydrogen chloride was excluded with continuous flow of nitrogen gas. The dry substance was dissolved in 10 ml of 0.1N hydrochloric acid and diluted with 0.2M sodium citrate buffer(pH 2.2). Under the conditions of the previous report(Park *et al.*, 1979) amino acids were analyzed with an Hitachi amino acid autoanalyzer Model KLA-5.

Standard amino acids were also analyzed under the same condition and a chromatogram was obtained. The amino acid mixture used for standardization contained 0.1 μmol of each amino acid(in case of proline, 0.2 μmol) dissolved in 0.5ml of 0.2M sodium citrate buffer(pH 2.2). Contents of each amino acid were calculated on the basis of the peak area by half width method.



Scheme I: Extraction procedure of the protein-polysaccharide fraction.



Scheme II: Antitumor test.

Results

1. Antitumor Test

From 100g of the dried carpophores of *Cryptoporus volvatus*, 9.3g of the protein-polysaccharide fraction was obtained. Antitumor effects of this fraction on

sarcoma 180 in mice were shown in Table I. Regression of the tumor was also examined on the 30th day after the tumor implantation. The tumor inhibition ratio of the high dose group was 80.4% and that of the low dose group was 70.3%.

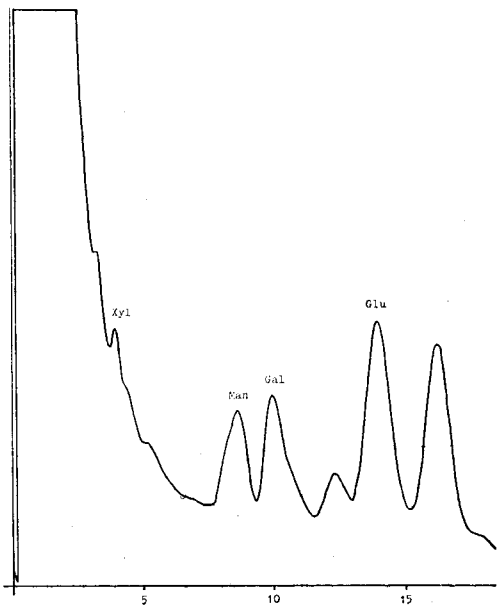


Fig. 2. G.L.C. pattern of the monosaccharides of the hydrolyzate of the polysaccharide fraction of ***Cryptoporus volvatus.***

2. Assay for Polysaccharide of the Antitumor Fraction

Total polysaccharide content of the fraction was 55.3%. Glucose, mannose, galactose, and xylose were identified as monosaccharide subunits of the fraction. Table II shows the percentage of the monosaccharide contents.

3. Assay for Protein of the Antitumor Fraction

Total protein content of the fraction was 18.2%. The chromatogram of amino acids of the fraction was shown in Fig. 4. Table III shows the percentage of each amino acid contained.

Table I. Antitumor activity of the protein-polysaccharide fraction of ***Cryptoporus volvatus.***

Group	Average tumor weight(g)	Inhibition ratio(%)	Complete regression
Control(8)[a]	5.51 ±1.11[b]	—	—
High Dose 50mg/kg(9)	0.88 ±0.37	84.0	5[c]
Low Dose 20mg/kg(8)	1.64 ±0.71	70.3	4

a: The number of mice used
b: Mean ± S. E. ($p<0.001$)
c: The number of mice in which 100% regression of the tumor was observed.

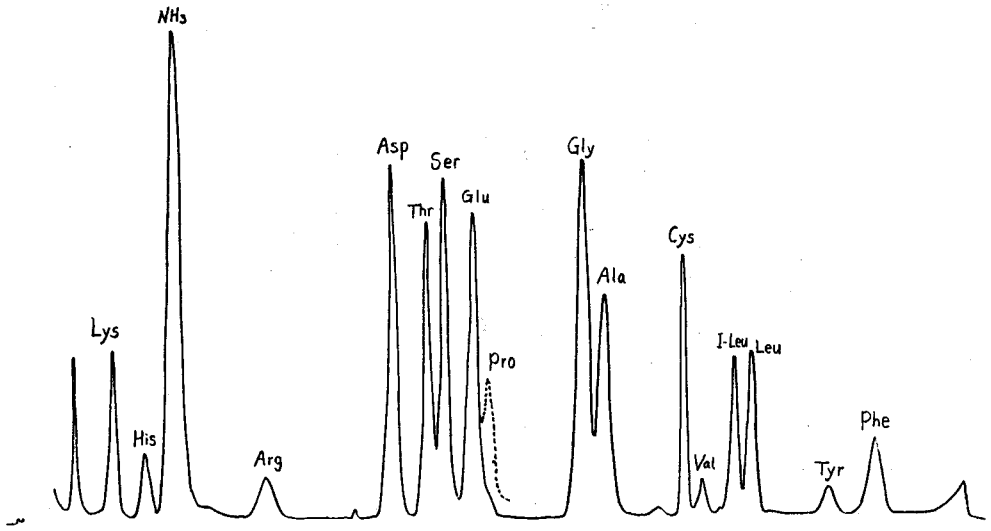


Fig. 3: Chromatogram of the amino acids of the protein moiety of the antitumor fraction of *C. volvatus.*

Table II. Contents of the monosaccharides of the polysaccharide in the antitumor fraction of *Cryptoporus volvatus*.

Tatal polysaccharide content=55%	
Monosaccharide subunit	Content(%)
Xylose	3.2
Mannose	23.1
Galactose	30.0
Glucose	43.7

Table III. The contents of amino acids of the protein in the antitumor fraction of *Cryptoporus volvatus*.

Amino acid	Content(%)
Lysine	3.5
Histidine	2.0
Arginine	2.6
Aspartic acid	10.6
Threonine	9.3
Serine	8.4
Glutamic acid	13.9
Proline	2.5
Glycine	10.3
Alanine	6.8
Cysteine	0.4
Valine	5.6
Methionine	8.3
Isoleucine	3.7
Leucine	6.1
Tyrosine	1.8
Phenylalanine	4.3

Discussion

When sarcoma 180 cells began to grow after they had been transplanted into A-strain mice, the mice were divided into three groups. The first group and second group were injected with the high and low doses of the protein-polysaccharide fraction of *Cryptoporus volvatus*, the third group receiving only the physiological saline as a control. In this control group the sarcoma grew steadily and became larger than those of the treated groups. The inhibition of the tumor growth in these mice as compared with that of the control mice was readily apparent in the inhibitio ratio data. This fraction exerted greater antitumor activity in the identical animal model than that of the carpophores of *Pleurotus ostreatus*(Kim *et al.*, 1979), which showed the inhibition ratio of 62.8% at the dose of 100mg/kg. However, it was less active than lentinan of *Lentinus edodes*(Maeda and Chihara, 1971) and PSK of *Coriolus versicolor*(Tsugagoshi and Ohashi, 1974).

The chemical analysis of the polysaccharide moiety of the antitumor fraction of *C. volvatus* showed that it contained glucose, galactose, mannose and xylose at a ratio of 44:30:23:3. That is, glucose was not the prevalent monosaccharide, whereas it was the major one in case of lentinan (86%), PSK (61%), *Russula pseudodelica* (74%) (Min *et al.*, 1979), *Microporus affinis* (75%) (Min *et al.*, 1979), *Schizophyllum commune* (62%) (Lee *et al.*, 1981), *Auricularia auricula-judae* (76%) (Lee *et al.*, 1981), and *Ganoderma lucidum* (73%) (Kim *et al.*, 1980). In the antitumor fraction of *Naematoloma fasciculare*, fructose, glucose, galactose, mannose and xylose were contained in the ratio of 29:26:24:19:1 (Lee *et al.*, 1981).

Contrary to the severe toxicities of the current antineoplastic agents, this antitumor fraction did not exhibit any acute toxicity in mice since no apparent toxic effect was observed in the organs of the mice. It is suggested that it may inhibit tumor growth by enhancing immunity against the tumor as previously shown in the polymers of other basidiomycetes but not by direct cytotoxicity to it (Namoto *et al.*, 1975; Kim *et al.*, 1979). Further studies on the separation of the protein and polysaccharide moieties and on the activity of each moiety are in progress.

In summary, the protein-polysaccharide fraction of *C. volvatus* inhibited the growth of sarcoma 180 in mice with no apparent acute toxicity and contained four monosaccharides and 18 amino acids.

Conclusion

The protein-polysaccharide fraction of *Cryptoporus*

volvatus showod high antitumor activity against sarcoma 180 implanted in mice. The antitumor fraction consisted of a polysaccharide (55.3%) and a protein (18.2%). The monosaccharides of the fraction were identified as glucose, galactose, mannose and xylose and 18 amino acids were identified.

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