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Antitumor Components of *Agrocybe cylindracea*

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To find pharmacologically active components of *Agrocybe cylindracea*, its basidiocarps were extracted with water. The extracts were separated by DEAE cellulose column chromatography, Sepharose CL-4B gel filtration, and Concanavalin A-Sepharose 4B affinity chromatography. Among the obtained fractions from *A. cylindracea*, fraction IN which was the neutral protein-bound-polysaccharide fraction exhibited a marked antitumor activity and it was tentatively named "Cylindan". It showed about 70% of tumor inhibition against the solid form of sarcoma 180 when a dose of 30 mg/kg/day was intraperitoneally injected into ICR mice. When each fraction was examined by chemical analysis, Cylindan consisted of 85% polysaccharide, 3% protein and 1% hexosamine. Its polysaccharide moiety contained glucose, mannose, fucose and galactose and its protein moiety contained the comparatively large amounts of aspartic acid and glycine, and other 11 amino acids.

Key words : Basidiomycete, *Agrocybe cylindracea*, Edible mushrooms, Protein bound-polysaccharides, Antitumor activity

INTRODUCTION

Many reports have shown that the extracts of Basidiomycetes are effective in inhibiting tumor growth (Kim *et al.*, 1979) and lowering blood cholesterol (Kim *et al.*, 1976). We had studied the antitumor activity of Basidiomycetes and reported that *Ganoderma lucidum* (Shim *et al.*, 1978), *Pleurotus pulmonarius* (Lee *et al.*, 1985), *Favolus alveorarius* (Chang *et al.*, 1988), *Volvariella bombycina* (Kim *et al.*, 1985), *Agaricus compestris* (Park *et al.*, 1985), *Lyophyllum descates* (Kim *et al.*, 1984), *Polyporus giganteus* (Kim *et al.*, 1987), *Coriolus versicolor* (Cho *et al.*, 1988), *Stropharia rugosoannulata* (Kim *et al.*, 1989) showed this activity. In this study we describe the antitumor activity of the basidiocarps of a mushroom, *Agrocybe cylindracea*, which has recently been cultivated by Agricultural Science Institute.

MATERIALS AND METHODS

The basidiocarps of *Agrocybe cylindracea* cultivated by Agricultural Science Institute were used. DEAE cellulose, Sepharose CL-4B, Concanavalin A-Sepharose 4B, Bovine serum albumin, and various monosaccharides were purchased from Sigma Chemical Co. (St. Louis, MO). Krestin as a standard in antitumor assay was products of Sankyo Co., Japan.

Extraction of mushroom components

The basidiocarps (5 kg) of *A. cylindracea* were washed 3 times and cut into small pieces, and 20 L of deionized water were added. After 5 hrs, extraction under refluxing at 85-90°C, the aquous extract was filtered through a 60 mesh filter. Further 10 L of deionized water were added to the filtrate and extracted again 2 times under the same condition. The filtrate was combined and condensed in vaccum. To the condensed aquous extract, 3 volume of cold 95% ethanol was added. After storage over-night at 4°C, it was centrifuged and the precipitate was collected. The obtained precipitate was dialyzed in visking tube in deionized water at 4°C for 7 days. After removing the precipitate by centrifugation, the supernatant was lyophilized and 10 g of brown powder was obtained. It was named Fr. CR. And the residual filtrate of the basidiocarps was extracted with 5% NaOH for 10 hrs at 20-30°C for 3 times. After removing the filtrates by filtration, the filtrate was neutralized by 1 N acetic acid and dialyzed. The inner fraction was lyophilized and 3 g of dark brown powder was obtained. It was named Fr. CA.

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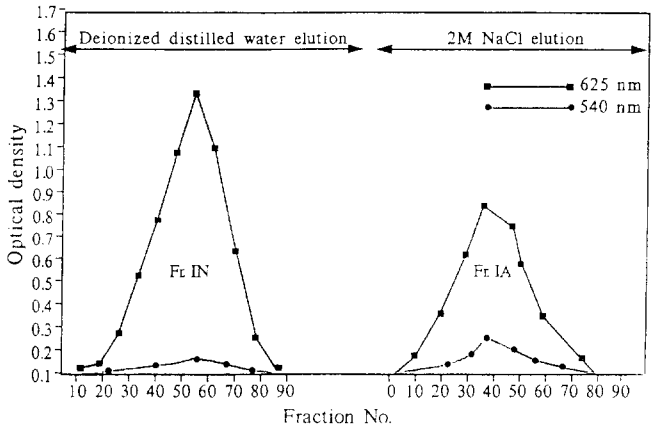

Fig. 1. Elution pattern of Fr. CR by DEAE cellulose anion exchange chromatography

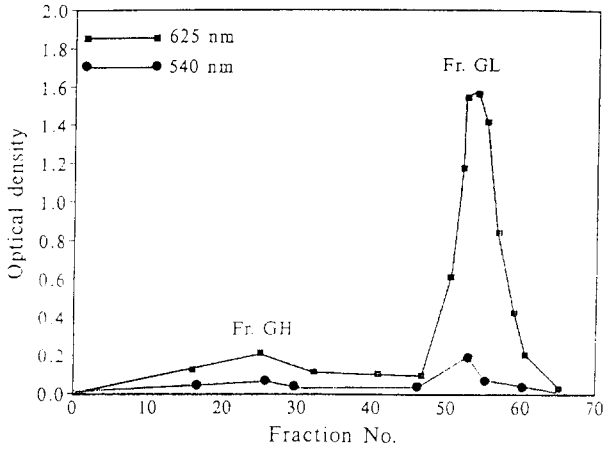

Fig. 2. Elution pattern of Fr. IN by Sepharose CL-4B gel filtration chromatography

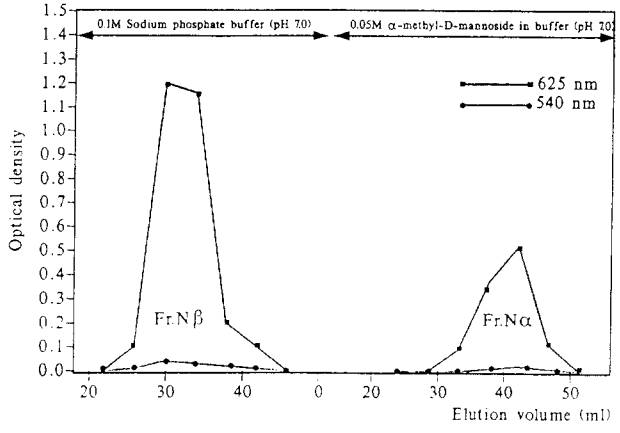

Fig. 3. Elution pattern of Fr. GL by Con A-Sepharose 4B affinity chromatography

Purification of antitumor components

DEAE cellulose ion exchange column chromatography : DEAE cellulose resin was used in the chloride form under the following conditions: column size, 3.2×60 cm, flow rate, 60 ml/hr. Fr. CR was dissolved in 50 ml of water and applied to a column of DEAE cellulose, then eluted with water (pH 7.2). Each 10 ml of effluent was collected into fractions. The positive fraction as shown by the anthrone test (625 nm) and Lowry-Folin test (540 nm), was collected, concentrated and lyophilized. The fraction obtained was a white powder (3 g) and it was designated Fr.- IN. The remaining materials on DEAE cellulose were eluted by 2 M NaCl. The positive fraction as shown by anthrone test was collected, concentrated, dialyzed and lyophilized. The fraction obtained was a brown powder (2 g) and was designated Fr. IA (Fig. 1).

Sepharose CL-4B gel filtration chromatography : Fr. IN (2 g) was dissolved in 0.01 M sodium phosphate buffer (pH 6.8) and applied to the prepacked column of Sepharose CL-4B (2.5×75 cm, Bio-Rad). It was eluted with the same buffer at a flow rate of 12-20 ml/hr and the effluents were fractionated into 4.8 ml/fraction. Optical densities were measured at 625 nm (Antrone test) and 540 nm (Lowry-Folin test) against each fraction. The anthrone positive fractions were collected and concentrated. The concentrate was dialyzed and lyophilized. The obtained fractions were designated Fr. GH (50 mg) and Fr. GL (1000 mg) (Fig. 2).

Concanavalin A-Sepharose 4B affinity chromatography : Fr. GL (800 mg) was dissolved in 0.1 M sodium phosphate containing 1 M NaCl (pH-7.0), and applied to the prepacked column of Con A-Sepharose 4B (2.0×30 cm, Bio-Rad). It was eluted with the same buffer at a flow rate of 30 ml/hr and the effluents unadsorbed were fractionated into 3.8 ml/fraction, and the adsorbed materials on Con A-Sepharose 4B were eluted by the solution of 0.05 M α-methyl-D-mannoside in 0.1 M NaCl (pH 7.0), and the Anthrone test and the Lowry-Folin test were done. The anthrone positive fractions were collected, concentrated, dialyzed and lyophilized. The unadsorbed fraction was designated Fr. Nβ (500 mg) and the adsorbed fraction was designated Fr. Nα (130 mg) (Fig. 3).

Assay of antitumor activity

In solid tumor : The mice used were males of the ICR strain obtained from the Experimental Animal Farm of Seoul National University, initially weighing about 20-25 g. All the mice kept on standard laboratory diet and water was provided ad lib. and they were divided into groups of 10 mice. Fresh ascites fluid containing approximately 5×10^5 cells of sarcoma 180 in 0.1 ml was implanted subcutaneously at the left groin of the mouse. Test samples dissolved in saline solution and the only saline solution in the negative control group and the Krestin

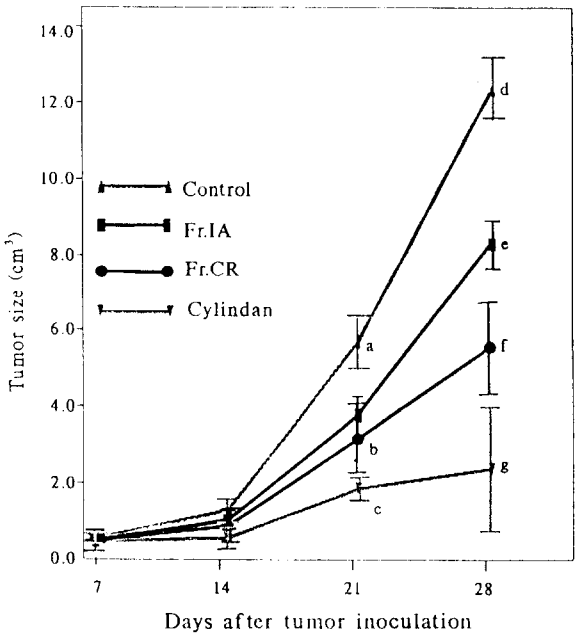

Fig. 4. Effects of antitumor components on sarcoma 180 solid tumor growth (a vs b, c and d vs e, f, g: $p<0.05$)

Table I. Effects of antitumor components on sarcoma 180 solid tumor

Fraction	Dose (mg/kg/day)	Tumor weight (g, Mean±S.E.)	Inhibition ratio(%)	ID_{50}* (mg/kg/day)
Control		6.60±0.91	—	
Krestin	20	2.71±0.45**	58.9	
CR	20	3.49±0.45	47.1	
	50	2.94±0.61	55.5	35
	100	2.54±0.43	61.4	
CA	20	5.04±0.74	23.6	
	50	4.78±1.44	27.8	>100
	100	3.57±0.38	45.9	
Control		8.67±1.30	—	
Krestin	20	3.17±0.94	63.0	
IN	3	5.40±0.57	37.7	
	20	3.90±1.17	55.0	14
	50	2.87±0.89	68.3	
IA	3	5.21±1.04	39.9	
	20	5.12±1.47	40.8	31
	50	3.84±0.69	54.7	
Control		5.13±1.09	—	
Nα	5	3.29±1.97**	35.8	
	15	3.26±1.02	36.5	>100
	30	3.12±0.46	39.2	
Nβ	5	2.06±0.81	59.8	
	15	1.21±1.04	76.4	6
	30	1.19±0.93	76.8	

*ID_{50} indicates dose level which inhibits tumor growth to 50% of the control.
**Duncan's test on all the data showed $p<0.05$.

dissolved in saline in the positive control group, were administered by intraperitoneal injection beginning day 3 after the implantation, once daily for 10 days.

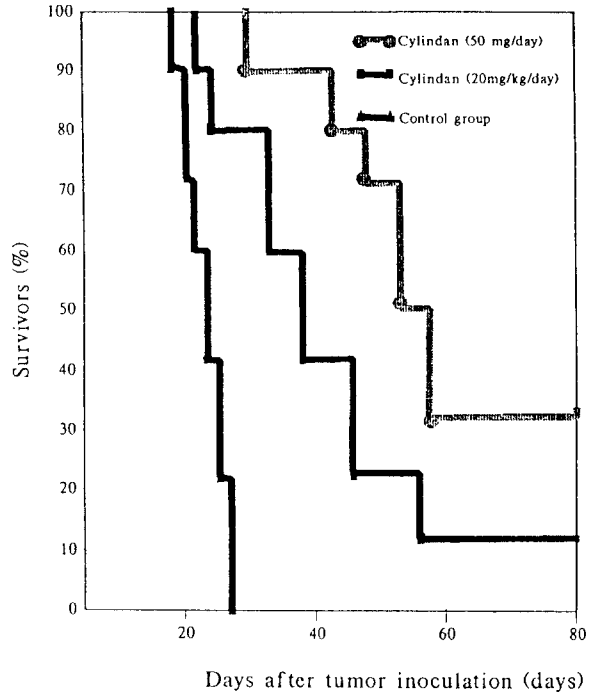

Fig. 5. Effects of Cylindan on the life span against the ascitic from of sarcoma 180 in ICR mice

The mice were killed day 28 after the implantation, and the tumor was dissected out and weighed to determine the inhibition ratios (Fig. 4). The inhibition ratios were calculated by the means of the following formula : inhibition ratio(%) = CW-TW/CW×100; where CW is the average tumor weight of the control group, and TW is that of the treated group. The results are shown in Table I.

In ascite tumor : After collection of the ascitic fluid from the mice bearing tumor cells, such as sarcoma 180, mouse Lewis lung carcinoma. Ehrlich carcinoma, mouse EL-4 leukemia, tumor cells were washed and diluted to 5×10^7 cells/ml and each 0.1 ml was inoculated into the mouse ascite. Each 0.1 ml of the test samples was intraperitoneally injected 24 hr. after tumor inoculation, once daily for 10 days, then mortality for 60 days were observed (Fig. 5, 6). The time and the percentage of survival were compared to the control group.

Chemical analysis

Protein content was determined by Lowry-Folin method (Cooper *et al.*, 1977) using bovine serum albumin (Sigma Co.) as a standard, and the polysaccharide content was determined by Herbert's method (Herbert *et al.*, 1971) using monosaccharides as a standard. The hexosamine content was quantitatively determined by Elson-Morgan method (Dische, 1962).

The sugar components : They were determined by

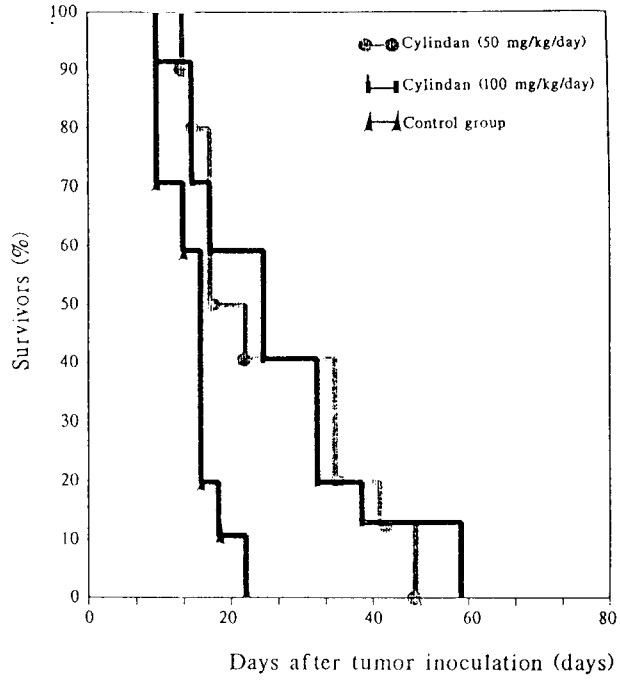

Fig. 6. Effect of Cylindan on the life span against the ascitic form of Lewis lung carcinoma in C57BL/6 mice

Mitruka method (Mitruka, 1975): that was, 5 mg of sample was dissolved in 3% HCl-methanol solution within a teflon cap tube. The HCl-methanol solution was obtained by adsorbing the gaseous HCl generating from the reaction of NaCl and conc. sulfuric acid into absolute methanol. After the air in the tube containing each sample and HCl-methanol was substituted with nitrogen gas, the tube was sealed. They were methanolized at 80±5°C for 20 hrs. The methanolysate was filtered, evaporated and dissolved in 1 ml of pyridine. For trimethylsilylation, 0.2 ml of hexa-methyldisilazane and 0.2 ml of trimethylchlorosilane were added to the solution and shaken vigorously. The sample solution was injected into a Hewlett 5890 Packard Series II gas chromatograph equipped with hydrogen flame ionization detector, using a glass column (0.2 mmϕ×2.5 m) packed with SE-54 at 180°C at a flow rate of 50 ml per min (nitrogen). Retention times and peak areas were measured by HP-3390 Integrator. Retention time of each peak was compared with that of standard monosaccharides. The contents of each monosaccharide were calculated from the chromatograms by measuring the peak area.

Amino acid analysis of the protein moiety of the antitumor components

5 mg of each sample was dissolved in 5 ml of 6 N HCl within the teflon cap tube. The air in the cap tube was substituted with nitrogen gas and the tubes were sealed. The samples in the tubes were hydrolyzed at 110±5°C for 24 hrs. After filtration, the filtrate was evaporated under reduced pressure and redissolved in 2 ml of 0.02 N HCl. The sample solutions were injected into the amino acid analyzer. Amino acid analysis was performed on a Hitachi amino acid analyzer 835 equipped with Tungsten detector, using an ion exchange column (No.2619 F, 4 mmϕ× 15 mm). The content of each amino acid was calculated from the chromatograms by peak area.

Table II. Effects of cylindan on ascitic forms of sarcoma 180, Lewis lung carcinoma, Ehrlich carcinoma and EL 4 leukemia

Tumor	Group	Dose (mg/kg/day)	Mean survival time (day, Mean±S.E.)	T/C (%)*
S180	Control		22.4±2.1[a]	100.0
	Cylindan	20	37.5±5.5[b]	165.6
		50	40.8±3.3[c]	182.3
LLC	Control		15.4±4.0[d]	100.0
	Cylindan	50	21.9±1.5	142.3
		100	24.9±4.8[e]	161.8
Ehrlich	Control	—	22.1±3.0	100.0
	Cylindan	50	24.7±2.8	116.0
EL4	Control	—	21.2±0.7	100.0
	Cylindan	50	21.0±1.4	99.0

$$*T/C\ (\%) = \frac{\text{Mean survival time of treated group}}{\text{Mean survival time of control group}} \times 100$$

(a) vs (b, c) and (d) vs (e) : $p<0.05$ (Duncan's test)

RESULTS AND DISCUSSION

The basidiocarps of *Agrocybe cylindracea* were extracted with water, 10 g of brown powder was obtained, and it was named Fr. CR. Fr. CR was applied to DEAE cellulose ion exchange column, then the unabsorbed fraction eluted by the deionized water, was named Fr. IN (3 g) which was a white powder of neutral polysaccharide. The absorbed fraction eluted by 2 M NaCl solution was named Fr. IA (2 g). Fr. IA was a brown powder of acidic polysaccharide. The elution pattern of Fr. IN and Fr. IA was shown in Fig. 1. After Fr. IN (2 g) was applied to Sepharose CL-4B gel filtration column, the fraction Fr. GH of high molecular weight and the fraction Fr. GL of low molecular weight were obtained. The elution profile was shown in Fig. 2. The Fr. GL (800 mg) was applied to Con A-Sepharose 4B affinity chromatography, the unadsorbed fraction (Fr. Nβ) which was β-glucan and the adsorbed fraction (Fr. Nα) which was α-glucan were obtained. The elution profile was shown in Fig. 3. Tumor inhibition ratios of each fraction and Krestin against solid and ascitic forms of tumor growth in ICR mice were shown in Tables I and II. Fr. Nβ showed the highest inhibition ratio of 77% among the fractions at a dose of 30 mg/kg/day and ID_{50} 6 mg/kg/day . The antitumor activity of Fr. Nβ was studied also by the prolongation of the life span

Table III. Polysaccharide, protein and hexosamine contents of the antitumor fractions of *Agrocybe cylindracea*

Fraction	Components (%)		
	Polysaccharide	Protein	Hexosamine
CR	81.6	13.5	2.20
CA	79.0	8.3	2.29
IN	85.6	2.7	1.13
IA	42.0	13.3	1.47
GL	88.0	2.6	1.28
Nα	82.4	4.7	1.32
Nβ	89.6	2.5	1.96

Table IV. Monosaccharide contents of the polysaccharide moiety of the antitumor fractions of *Agrocybe cylindracea*

Fraction	Contents (%)					
	Glucose	Galactose	Mannose	Fucose	Xylose	Ribose
CR	74.6	8.7	6.4	5.9	3.3	1.0
CA	93.6	1.6	3.3	0.6	N.D.	0.7
IN	24.9	27.6	18.0	12.1	6.8	10.5
IA	81.2	5.4	9.3	1.4	N.D.	0.6
GL	36.9	22.2	10.5	17.9	10.2	2.2
Nα	31.8	5.9	47.3	6.5	8.4	N.D.
Nβ	31.6	35.7	1.3	12.2	13.1	6.1

ND means not detected.

Table V. Amino acid contents of the protein moiety of the antitumor fractions of *Agrocybe cylindracea*

Amino acid	Contents (%)						
	Fr.CR	Fr.CA	Fr.IN	Fr.IA	Fr.GL	Fr.Nα	Fr.Nβ
Aspartic acid	10.7	8.6	13.8	13.5	5.1	6.4	6.7
Glutamic acid	7.8	8.4	3.6	10.2	6.3	7.7	7.6
Threonine	5.1	3.8	7.4	6.3	4.0	4.9	3.3
Serine	6.5	6.4	ND	8.3	6.4	8.7	4.9
Glycine	12.1	10.7	10.2	12.1	10.2	12.1	11.3
Alanine	9.3	8.8	8.8	ND	9.2	10.0	10.7
Valine	4.8	6.4	6.3	6.9	4.7	5.4	6.6
Methionine	1.0	1.3	1.3	1.3	2.6	0.6	1.0
Isoleucine	1.3	1.7	1.5	1.7	1.3	1.5	1.5
Leucine	3.1	5.2	3.09	4.1	4.1	4.5	4.6
Tyrosine	1.6	2.1	0.99	1.8	1.2	ND	1.5
Pheylalanine	3.5	3.7	4.49	4.0	6.4	3.8	5.7
Lysine	3.5	2.8	1.65	2.3	3.0	2.7	2.8
Histidine	1.2	1.7	ND	1.3	0.8	1.3	0.8
Arginine	1.2	1.7	1.00	1.0	1.1	1.7	1.6

ND means not detected.

of mice bearing ascitic tumor. It was found that when 50 and 100 mg/kg/day of Fr. Nβ were respectively administered to the mice bearing sarcoma 180 ascitic tumor, Lewis lung carcinoma intraperitoneally, the survival of the mice increased. But the statistically significant prolongation of life span of the mice bearing Ehrlich carcinoma and EL-4 leukemia were not observed. Although data are not shown here, Fr. Nβ did not exhibit significant inhibition to solid form of Lewis lung carcinoma. Thus the protective effects of Fr. Nβ on tumors are considered to be dependent upon the type of the tumor cells. The contents of polysaccharides and protein, hexosamine of each fraction were shown in Table III. The monosaccharides of the polysaccharide moiety in Fr. IN were glucose, mannose, fucose and galactose (Table IV) and the protein moiety contained 13 amino acids, such as valine, aspartic acid, and glutamic acid, as shown in Table V. Thus the protein, the polysaccharide and a small quantity of hexosamine were detected. The polysaccharides obtained from the fruiting body of *Agrocybe cylindracea* are considered to be a protein-bound polysaccharide.

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