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Inhibitory activities of polysaccharide purified from *Phellodendron chinese* SCHNEID on melanoma B-16-derived metastatic tumor and hypersensitivity

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SUMMARY

The polysaccharide fractions were isolated and purified from *Phellodendron chinese* SCHNEID, and antitumor activities using the melanoma B-16-derived metastatic tumors were examined at dosages of 2, 5 and 10 mg/100 g. F-7 and F-8 showed the highest tumor metastatic inhibitory activities (inhibition ratio 60 and 80% in 2 mg/100 g), and in dose of 5 mg/100 g, the inhibitory ratios were 85 and 70%, respectively. Furthermore, 10 mg/100 g of intraperitoneal (*i.p.*) injection gave 90 and 95% of inhibition. When the effects of polysaccharides on hypersensitivity were examined, the inhibitory activities were not markedly observed in oral administration, indicating that the polysaccharides are directly acting to immune system. Also, the polysaccharides increased the number of circulating blood leukocytes and total peritoneal exudate cells. Although implantation of tumor cells greatly decreased the productivity of antibody (antibody-mediated) and T lymphocyte reactivity (delayed-type) as 6.3 from 9.3 and 5.9 from 7.7, represented by the increase of footpad thickness, respectively. the polysaccharides elevated the reactivity of T lymphocyte in tumor-bearing mice, which were rapidly recovered by discontinuance of sample treatments. Especially, F-2, F-5, F-7 and F-8 remarkably recovered the decreased sensitivity. These results clearly indicated that the *i.p.* injection is much effective to suppress tumor growth than oral administration.

INTRODUCTION

Key words : *Phellodendron chinese* SCHNEID; Polysaccharide; Immunopotentiation; Antitumor activity.

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Various kinds of polysaccharides from oriental herbs were reported to exhibit diverse biological activities, especially including the activities on immune system and cancer. It was also previously reported that some polysaccharides isolated from bamboo leaves, bagasse, were remarkably effective in inhibiting the growth and inducing regression of sarcoma-180 solid tumor subcutaneously transplanted in mice

(Ikekawa *et al.*, 1968). This tumor-inhibiting effect was considered to be indirect and host-mediated, and not due to their cytocidal action on tumor cells. With respect to application of natural medicinal herbs in cancer, many polysaccharides extracted from Basidiomycetes have also been reported to act as biological response modifiers (BRM) (Tanaka *et al.*, 1965; Hamuro *et al.*, 1978; Maeda and Chihara, 1971; Komatsu *et al.*, 1969; Oh *et al.*, 1992; Han *et al.*, 1995).

Those facts led us to characterize the active principle from the extracts guided by an tumor inhibitory assay system and immunepotentiation assay system. As the continuation of the works to clarify such activities, chemical and biological studies were attempted. In this study, *Phellodendron chinese* SCHNEID polysaccharides were isolated and purified with water and alkali. It was confirmed that the polysaccharides of the *Phellodendron chinese* SCHNEID showed strong immunopotentiating and anti-tumor activity against melanoma B-16-derived metastatic tumors in mice.

MATERIALS AND METODS

Animals and Materials

Female (C57BL/6XC3H) F1 (B6C3F1) mice maintained in Korea Research Insitute of Bioscience and Biotechnology, KIST (Taejon, Korea) were used in all experiments. The 17-22 g mice were used as the source of the spleen cells. Sheep red blood cells (sRBCs) were obtained from Korea Media Co., Ltd. (Seoul, Korea). Guinea pig complement and RPMI 1640 were purchased from Gibco BRL (Grand Island, NY, USA). *Phellodendron chinese* SCHNEID was obtained from Dongguk University Oriental Medical Hospital (Kyungju, Korea). Melanoma B-16-derived metastatic tumor cells were our deposit. Lipopolysaccharide (LPS), dimethylsulfoxide (DMSO) and Proteinase K (from *Tritirachium album*) were purchased from Sigma (St. Louis, MO, USA).

General analytical methods

Total sugar content was measured by the Phenol-sulfuric acid method (Dubois *et al.*, 1956). Reducing sugars were measured by the Nelson-Somogyi method (Miller, 1959)

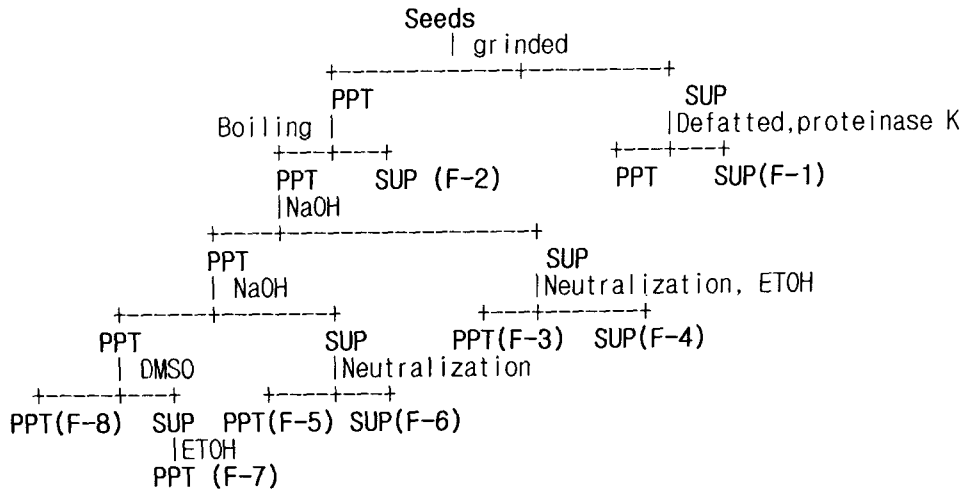

Fig. 1. Summary of fractionation of *Phellodendron chinese* SCHNEID polysaccharides

sing glucose (Glc) as a standard. Protein was determined by the procedure of Lowry *et al.* using bovine serum albumin (BSA) as a standard. Hydrolysis of the polysaccharide was usually done by heating with 2 M sulfuric acid at 100℃ for 6 h in a screw-capped vial. Following neutralization of the hydrolyzate with barium carbonate ($BaCO_3$) and filtration of the precipitated BaSO4, the filtrate was passed through an Amberite IR-120 (H^+ form) column and the acidic solution evaporated to a syrup.

Hydrolysis of the polysaccharides and Determination of molecular weights of the polysaccharides

Partial hydrolysis of the polysaccharide was done with 25 mM sulfuric acid (10 ㎖), at 100℃, for 4 h to remove only the galactofuranosyl residues. The hydrolysate was neutralized with 0.5 M NaOH and dialyzed against distilled water. Gas-liquid chromatography (GLC) was done with a Hitachi gas chromatography model 163 for neutral sugars fitted with a flameionization detector. Sugars were separated on a column (0.4 × 200 cm) of 3% ECNSS-M on Gas Chrom Q, at 190℃.

The molecular weights of the polysaccharides were estimated by gel filtration using a Sepharose CL-6B column (3.0 × 100 cm of Bio-Rad Co.) eluted with 0.1 M NaOH, at a flow rate of 12.5 ml/h, compared with pullulans (5×10^3 2×10^5, Hayashibara Biochemicals Lab. Inc., Okayama, Japan) and dextrans (2×10^4-2×10^6, Sigma) as authentic standards of molecular weight.

Extraction, fractionation, and purification

Polysaccharides were prepared by repeated ethanol precipitation and dialysis against water followed by lyophilization. The dried samples (50 g) were homogenized using a mechanical disintegrator with Tekmar Tissuehomogenizer (Tekmar Co., Cincinati, OH, USA) in water, collected by centrifugation (15,000 × g, for 20 min) at 4℃, and delipidated with mixtures of chloroform and methanol (2:1) and (1:1) (delipidated fraction, yield 7.2 g). The delipidated fraction (7.2 g) was digested with proteinase K in 50 mM phosphate buffer (pH 7.2) containing 15 mM $CaCl_2$, at 37℃, for 48 h, centrifuged, and the supernatant solution was concentrated to about 12 ml, and added to ethanol (3 vol) resulting in precipitation of a polysaccharide (F-1, yield 5.2 g). The residue from the centrifugation was extracted three times with hot water at 120℃ for 20 min. The extracts were combined, concentrated, and poured into ethanol (4 vol) to give a hot water-extractable polysaccharide (F-2, yield 0.68 g). The residue after extraction was treated three times with 1 M NaOH at 20℃ for 6 h each, under a nitrogen atmosphere in the pressure of a small amount of sodium borohydride. This cold alkali extracts were combined, neutralized with 1 M acetic acid, and dialyzed against water. A part of the retentate was insolubilized for dialysis (F-3, yield 0.41 g). The soluble fraction was precipitated by the addition of ethanol (F-4, yield 0.24 g). The residues was treated three times with 1 M NaOH at 60℃ for 6 h, in a similar fashion. From the hot alkali extract, two polysaccharide fractions (F-5, 0.14 g; F-6, 0.1 g) were prepared in the same manner as that for F-3 and F-4, respectively. The alkali insoluble residue was extracted with DMSO at 60℃ overnight. The extracts were dialyzed against water. The retenate was precipitated by the addition of ethanol (F-7, 0.3 g), and the residue after extraction was washed with water (F-8, 1.1 g).

Antitumor activity using melanoma B-16-derived metastatic tumor

Assay of the antitumor activities of glucans of the extracts were done by the procedure

of Misaki *et al.* Four-week-old female ICR mice were obtained from KRIBB, KIST (Taejon, Japan). melanoma B-16-derived metastatic tumor cells (0.1 ml, 7 × 10^6 cells) were transplanted subcutaneously and tain vein injection into the right groins of the mice. The test samples, dissolved or suspended in PBS (10 mM, pH 7.0) in adequate concentrations, were autoclaved and injected intraperitonearlly daily for 10 days (injection volume, 0.1 ml), starting 24 h after tumor implantation. All mice were kept under observation for 5 weeks and then killed for final evaluation of the effects of treatment on tumor growth. Tumor numbers were counted. The growth inhibition ratios were calculated by the following equation.

Inhibition ratio (%) = 100 (A-B)/A

where A is the average tumor colony number of the control group and B is that of mice treated group. Complete regression indicates the ratio of the number of mice showing complete regression to the number of mice tested (Kataoka *et al.*, 1994).

Enzyme preparation and assay

All specimens of tumors removed and stored in each mouse were pulverized with an autopulverizer under liquid nitrogen and homogenized with 10 volumes of 5 mM Tris-HCl buffer (pH 7.5), containing 0.1 mM EDTA, 1 mM β -mercaptoethanol and 0.25 M sucrose, at final concentration, at 0℃. The homogenate was centrfuged for 1 h at 4℃ at 105,000 × g, and the supernatant was used as the crude enzyme preparation. As previously reported by Sakamoto *et al.* activitiesn of thymidylate synthetase (TS) and thymidine kinase (TK) were determined by the methods of Dunlap *et al.*, and Taylor *et al.*, respectively. Enzyme activity was normalized to tissue protein content and expressed as fmol/mg protein/min. Values were means of duplicate assays.

Activities of the polysaccharides on immune responses

To determine whether the purified polysaccharides have the immunopotentiating activities , the numers of circulating leukocytes (Miruka and Rawnsley, 1981) and total peritoneal cells (Weir, 1973) were measured after treatment of the polysaccharides to tumorbearing mice. Also the effects on antibody-mediated and delayed-type hypersensitivities in tumor-bearing mice were accompanied following the same methods as described previously (Katsura *et el.*, 1977; Titus and Chiller, 1981; Henningsen, 1984).

Statistical analysis

The statistical significance of difference among groups was evaluated by Student's t-test or Duncan's new multiple range test; $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

Isolation, purification, composition and molecular weight of the polysaccharides from *Phellodendron chinese* SCHNEID

In a preliminary study, water extracted fraction showed a potent growth-inhibitory activity against implanted in mice Sarcoma 180 solid tumor, therefore, polysaccharides were further purified by gel permeation chromatography using TSK Gel HW50S (Choi *et al.*, 1997). The purified polysaccharides was composed of GlcNAc (47.3%), Gal (24.7%) and Man (28.0%). Its optical rotation ($[\alpha]^{25}_D$ +43° , c, 0.60, 1 M NaOH) suggested that it contains β -glycosidic linkages. Table 1 shows their neutral carbohydrate compositions, as analyzed by GLC. It was apparent that F-1 solubilized from the extracts by treatment of Proteinase K constituents of GlcNAc, Gal and Man in a higher proportion than that of Glc. The test treated with Fehling reagent showed that is did not give an

insoluble copper-hydroxide complex, indicating that the Man-rich fraction does not contain mannan as a homopolysaccharide. All fractions were further applied to a gel permeation column and then eluted as described in Materials and Methods. As shown in Table 1, molecular weights of the fractions were determined by comparison molecular weight markers.

method described in Materials and Methods The results were shown in Table 2. Among the polysaccharide fractions, the water extracted fractions, particularly F-2, showed a potent growth-inhibitory activity against implanted in mice melanoma B-16-derived metastatic tumor (inhibition, 80%, at 2 mg/100 g), but the cold alkali extract (F-4) showed a very low acticity (inhibition; 5%) (Table 2). On the other

Table 1. Summary of physicochemical properties of the polysaccharides

Fraction	Molar ratio[a]				Molecualr weight	Protein
	GlcNAc	Gal	Man	Glc	(kDa)	(%)
F-1	4.1	1.0	2.6	0.6	1.5×10^6	1.5
F-2	1.4	1.0	0.9	5.6	5.2×10^5	1.0
F-3	0.7	1.0	5.1	4.2	3.1×10^4	1.2
F-4	2.4	1.0	4.4	1.1	3.4×10^5	0.3
F-5	3.7	1.0	3.2	2.0	1.3×10^4	1.9
F-6	8.7	1.0	4.2	0.3	2.4×10^5	1.2
F-7	2.1	1.0	6.4	1.7	1.6×10^4	0.1
F-8	0.5	1.0	1.9	8.3	5.2×10^3	0.4

[a]Measured by as their alditol acetates

Antitumor activities of the polysaccharides
To clarify the mode of action in antitumor activities of crude polysaccharides in a preliminary study, purified polysaccharides were applied to solid tumor in mice. Antitumor activities of polysaccharides were examined in a system using melanoma B-16-derived metastatic tumors implanted in ICR mice by intraperitoneal injection at dosages of 2 mg/100 g, 5 mg/100 g and 10 mg/100 g for 10 days, starting one day after tumor implantation (Gorin, 1985), by the hand, F-7 and F-8 showed the highest tumor inhibitory activities of inhibition ratio 90 and 85% in dose of 2 mg/100 g, respectively. In dose of 5 mg/100 g, the inhibitory ratios were 90% and 92%, respectively. When 10 mg/100 g was i.p. injected to the mice, 90 and 80% of inhibitory effects were observed. However, when they were orally administrated, the inhibitory activities were not markedly observed, indicating that the polysaccharides are directly acting to immune system.

Table 2. Inhibitory of anti-metastatic activities against Melanoma B-16[a] of the polysaccharides

Fraction	Dose (mg/100g×10days)	Tumor colony (No)	Inhibition ratio (%)
F-1	2.0	7.3±1.5	46.4
	5.0	7.6±1.4	45.4
	10.0	7.1±1.3	44.7
F-2	2.0	0.4±0.3	99.3
	5.0	1.2±0.2	76.4
	10.0	1.2±0.2	90.5
F-3	2.0	3.0±0.7	63.7
	5.0	5.3±0.6	62.7
	10.0	3.0±0.4	72.9
F-4	2.0	15.3±2.4	6.2
	5.0	21.1±1.1	4.1
	10.0	10.1±1.2	21.5
F-5	2.0	3.0±0.6	85.7
	5.0	4.5±0.2	57.4
	10.0	3.2±0.3	72.6
F-6	2.0	6.9±0.8	32.4
	5.0	2.3±0.2	34.6
	10.0	8.2±0.8	47.3
F-7	2.0	1.4±0.1	76.8
	5.0	1.2±0.1	55.5
	10.0	1.3±0.1	76.7
F-8	2.0	1.4±0.8	78.8
	5.0	0.5±0.1	67.1
	10.0	0.3±0.6	68.2
Control	-	24.1±1.5	-

[a]Tumor colony numbers, melanoma B-16-derived metastatic tumor, average ± S.D.

Effects of polysaccharides on immune function

Polysaccharides isolated were subjected to the immunopotentiating assays using the methods of comparing the changes in numbers of blood leukocytes and peritoneal exudate cells. All the polysaccharide fractions markedly increased the number of cells which are important marks of immune system and such increments were greater

Table 3. Effects of purified polysaccharides on the number of leukocytes in blood and peritoneal exudate cells

Treatments[a]	Cell number (mean±S.E.)			
	Leukocytes (10^3 cells/mm^3)		Exudate cells (10^5 cells/ml)	
	Day 1[b]	Day 10	Day 1	Day 10
Control	9.31±1.43	9.54±1.54	8.4±1.5	12.5±1.5
F-1	8.34±1.23	9.60±1.53	14.4±1.8	14.6±1.4
F-2	12.58±1.44	12.76±1.21	18.6±2.1	16.3±2.3
F-3	9.54±1.54	10.93±1.15	15.3±1.7	13.4±1.2
F-4	9.64±1.25	10.16±1.66	12.4±1.0	12.8±2.2
F-5	11.43±1.54	13.57±1.36	21.5±2.5	15.2±2.3
F-6	9.54±1.34	9.67±1.26	17.2±2.2	13.2±1.6
F-7	11.17±1.23	12.65±1.42	21.4±2.4	17.3±1.5
F-8	10.65±1.44	11.34±1.25	16.3±1.8	13.4±3.4

[a]Samples were injected 5 mg/kg, i.p., once a day for 10 consecutive days to each group which consisted of nine ICR mice for leukocytes and exudate cells. Control group (ten mice) were treated with saline buffer only.
[b]Days after the final treatment of sample

along the repeated treatments of sample within ten days (Table 3). The numbers of cells were recovered after seventh day to 10th day of final treatments.

Effects of polysaccharides on hypersensitivity

The effects of polysaccharides on hypersensitivity are summarized in Table 4. Implantation of tumor cells greatly decreased the productivity of antibody (antibody-mediated) and T lymphocyte reactivity (delayed-type) as 6.3 from 9.3 and 5.9 from 7.7, represented by the increase of footpad thickness, respectively. In the conditions, polysaccharide F-2, F-5, F-7 and F-8 remarkably recovered the decreased sensitivity.

Table 4. Effects of purified polysaccharides on the antibody-mediated hypersensitivity (AMH) and delayed-type hypersensitivity (DTH) in tumor bearing mice represented by the increase of footpad thickness

Treatment[a]	Increased footpad thickness[b]	
	AMH	DTH
Normal	9.3	7.7
S-180 bearing	6.3	5.9
F-1	5.9	6.0
F-2	8.8	7.2
F-3	7.2	6.9
F-4	7.7	6.3
F-5	8.1	7.2
F-6	6.7	6.1
F-7	8.7	7.3
F-8	7.8	6.7

[a]Samples were injected 5 mg/kg. i.p., once a day for 10 consecutive days to each group which consistes of nine ICR mice. Each ten of normal and tumor-bearing mice were treated with saline buffer only.
[b]Value in 1/10 min.

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