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Effects of polysaccharide fractions from phellodendron chinese SCHNEID on tumor progression and immunopontentiation

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Runnig title: Immunopontentiating and antitumor activities of polysaccharide

SUMMARY

In the previous paper (Kim *et al.*, Glycoconjugate Journal 16, 247-252, 1999), heteropolysaccharides from Korean medicinal plant, Phellodendri cortex (Hwangbek) showed a poten B-lymphocyte-stimulating activity in a system using polyclonal antibody forming cells in C57BL/6XC3H mice at dosages of 2-10 mg. In a series of biolgical active polysaccharides from natural medicinal plants, the polysaccharide fractions were isolated and purified from Phellodendron chinese SCHNEID, and antitumor activities were examined at dosages of 2, 5 and 10 mg/100 g. F-7 and F-8 showed the highest tumor inhibitory activities (inhibition ratio 96.4 and 98.2% in 2 mg/100 g), and in dose of 5 mg/100 g, the inhibitory ratios were 95.3 and 97.5%, respectively. Furthermore, 10 mg/100 g of intraperitoneal (i.p.) injection gave 97.3 and 98.7% of inhibition. In oral administration, the inhibitory activities were not markedly observed, indicating that the polysaccharides are directly acting to immune system. When the effects on TS and TK activities were determined, TS activities in the F-2 and F-7-treated mice were markedly suppressed to 73.7% and 79.5% of that in the control ($p<0.01$), while there was little difference in TK activity with a slight decrease in F-2 only. However, in i.p. injection, TS activities in the F-2, F-5, F-7 and F-8-treated mice were markedly suppressed to 83% to 85% of that in the control ($p<0.01$). Furthermore, there was also significant differences in TK activities in F-2, F-5, F-7 and F-8-treated mice ($p<0.05$). Therefore, polysaccharide fraction F-8 was further purified to active fractions of F-9 and F-11 by gel permeation chromatography using TSK Gel HW50S. The purified polysaccharides of F-9 and F-11 were composed of GlcNAc (47.3%), Gal (24.7%) and Man (28.0%). These results clearly indicated that the i.p. injection is much effective to suppress tumor growth than oral administration.

Keywords: Phellodendron chinese SCHNEID; Polysaccharide; Immunopotentiation; Tumor progression
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INTRODUCTION

It was reported that some polysaccharides isolated from plant leaves were remarkably

effective in inhibiting the growth and inducing regression of sarcoma-180 subcutaneously transplanted in mice (Han *et al.*, 1995). For theoretical explanation of these anti-tumor activities, Oh *et al.* (1992) have shown that the immune-stimulating activity of polysaccharides from Phellinus linteus might be associated with a functional stiumulation of B-lymphocytes, resultng in cancer prevention (Oh *et al.*, 1992).

Many 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; Ikekawa *et al.*, 1968; Hamuro *et al.*, 1969; Maeda and Chihara, 1971; Komatsu *et al.*, 1978; Oh *et al.*, 1992).

Those facts led us to characterize the active principle from the extracts guided by an tumor inhibitory assay system and immunepotentiation assay system (Miruka and Rawnsley, 1981). 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 sarcoma-180 solid tumors in mice.

MATERIALS AND METHODS

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). Lipopolysaccharide (LPS), dimethyl-sulfoxide (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) sing glucose (Glc) as a standard. Protein was determined by the procedure of Lowry *et al.* (1951) 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 $BaSO_4$, the filtrate was passed through an Amberite IR-120 (H+ form) column and the acidic solution evaporated to a syrup (Miruka and Rawmsley, 1981).

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 ml), 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. Gasliquid chromatography (GLC) was done with a Hitachi gas chromatography model 163 for neutral sugars fitted with a flame-ionization 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 (Miruka and Rawmsley, 1981).

Extraction, fractionation, and purification

Polysaccharides were prepared by repeated ethanol precipitation and dialysis against water followed by lyophilization. The dried samples (50g) 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 Sarcoma S-180

Assay of the antitumor activities of glucans of the extracts were done by the procedure of Misaki *et al.*(1986). Four-week-old female ICR mice were obtained from KRIBB, KIST (Taejon, Japan) Sarcoma 180 ascites cells (0.1 ml, 7×10^6 cells) were transplanted subcutaneously 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. Tumors were excised and weighed. The growth inhibition ratios were calculated by the following equation.

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

where A is the average tumor weight 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-Shirasygi *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 hour at 4℃ at 105,000×g, and the supernatant was used as the crude enzyme preparation. As previously reported by Sakamoto *et al.* (1987) activitiesn of thymidylate synthetase (TS) and thymidine kinase (TK) were determined by the methods of Dunlap *et al.* (1971) and Taylor *et al.* (1971) respectively. Enzyme activity was normalized to tissue protein content and expressed as fmol/mg protein/min. Values were means of duplicate assays.

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

1. 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 fractionated as shown in Fig. 1 (Choi *et al.*, 1997).

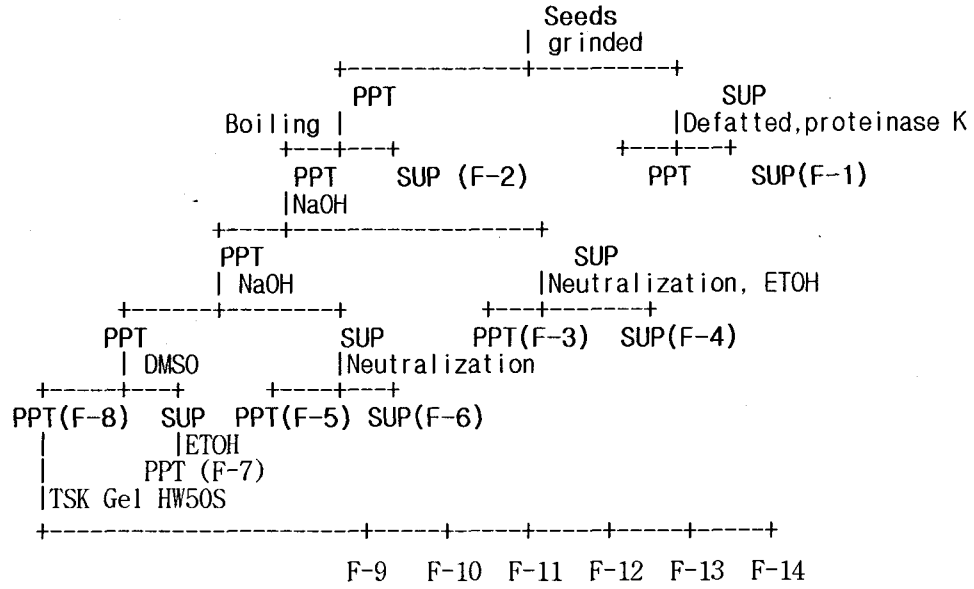

Fig. 1. Summary of fractionation and purification of Phellodendron chinese SCHNEID polysaccharides.

The purified polysaccharides was composed of GlcNAc (47.3%), Gal (24.7%) and Man (28.0%). Its optical rotation ([α]25D + 43° , c, 0.60, 1 M NaOH) suggested that it contains β -glycosidic linkages. Table 1 shows their neutral carbohydrate composi-

tions, 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 with molecular weight markers.

Table 1. Summary of physicochemical properties of the polysaccharides.

Fraction	Molar ratio[a]				Molecualr weight (kDa)	Protein (%)
	GlcNAc	Gal	Man	Glc		
F-1	4.1	1.0	2.6	0.6	1.5 × 106	1.5
F-2	1.4	1.0	0.9	5.6	5.2 × 105	1.0
F-3	0.7	1.0	5.1	4.2	3.1 × 104	1.2
F-4	2.4	1.0	4.4	1.1	3.4 × 105	0.3
F-5	3.7	1.0	3.2	2.0	1.3 × 104	1.9
F-6	8.7	1.0	4.2	0.3	2.4 × 105	1.2
F-7	2.1	1.0	6.4	1.7	1.6 × 104	0.1
F-8	0.5	1.0	1.9	8.3	5.2 × 103	0.4
F-9	0.5	1.0	1.9	8.3	5.2 × 103	0.4
F-11	0.5	1.0	1.9	8.3	5.2 × 103	0.4

[a] Measured by as their alditol acetates

2. 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 Sarcoma 180 solid 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 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 Sarcoma 180 solid tumor (inhibition, 95.3%, at 2 mg/100 g), but the cold alkali extract (F-4) showed a very low acticity (inhibition; 4.7%) (Table 2). On the other hand, F-7 and F-8 showed the highest tumor inhibitory activities of inhibition ratio 96.4 and 98.2% in dose of 2 mg/100 g, respectively. In dose of 5 mg/100 g, the inhibitory ratios were 95.3 and 97.5%, respectively. When 10 mg/100 g was i.p. injected to the mice, 97.3 and 98.7% of inhibitory effects were observed. Also, F-9 and F-11, which were purified by gel filtration, showed the excellent tumor inhibitory activities of inhibition ratio 97.1 and 98.3% in dose of 2 mg/100 g, respectively However, when they were orally administrated, the inhibitory activities were not markedly observed, indicating that the polysaccharides are directly acting to immune

Table 2. Activities against Sarcoma 180[a] of the polysaccharides.

Fraction	Dose (mg/100g × 10 days)	Tumor weight (g)	Inhibition ratio (%)	Complete regression
F-1	2.0	6.65 ± 1.55	44.7	1/10
	5.0	6.32 ± 1.43	44.7	1/10
	10.0	6.12 ± 1.35	44.7	1/10
F-2	2.0	0.57 ± 0.36	95.3	9/10
	5.0	0.65 ± 0.24	94.2	8/10
	10.0	0.54 ± 0.25	95.7	8/10
F-3	2.0	3.71 ± 0.76	69.2	4/10
	5.0	4.10 ± 0.67	63.2	3/10
	10.0	3.73 ± 0.45	70.9	4/10
F-4	2.0	11.47 ± 1.43	4.7	0/10
	5.0	11.23 ± 1.12	4.9	0/10
	10.0	9.43 ± 1.23	14.3	1/10
F-5	2.0	2.98 ± 0.65	75.2	5/10
	5.0	2.65 ± 0.23	77.3	5/10
	10.0	2.23 ± 0.32	82.1	7/10
F-6	2.0	6.91 ± 0.87	42.6	1/10
	5.0	6.63 ± 0.95	44.3	0/10
	10.0	6.25 ± 0.85	47.3	1/10
F-7	2.0	0.43 ± 0.12	96.4	8/10
	5.0	0.45 ± 0.09	95.3	8/10
	10.0	0.34 ± 0.11	97.3	9/10
F-8	2.0	0.21 ± 0.10	98.2	9/10
	5.0	0.32 ± 0.05	97.5	9/10
	10.0	0.33 ± 0.07	98.7	8/10
F-9	2.0	0.22 ± 0.11	96.5	9/10
	5.0	0.33 ± 0.06	97.1	9/10
F-11	2.0	0.24 ± 0.05	97.7	9/10
	5.0	0.30 ± 0.04	98.3	9/10
Control	-	12.04 ± 1.52	-	0/10

[a] Tumor weight, Sarcoma 180 solid tumor, average ± S.D.

system.

3. Effects of the polysaccharides on TS and TK activities in tumor mice when orally administrated or i.p. injected.

To measure the enzyme activities in tumor specimens which were removed from the orally administrated or i.p. injected mice, 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 centrifuged for 1 h at 4℃ at 105,000 × g, and the supernatant was used as the crude enzyme preparation. Then, activities of TS and TK were determined. In orally administrated mice, TS activities in the F-2 and F-7-treated mice were markedly suppressed to 73.7% and 79.5% of that in the control ($p<0.01$ by Student's t-test) (Table 3), while there was little difference in TK activity with a slight decrease in F-2 only. However, in i.p. injection, when 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 and control group (ten mice) were treated with saline buffer only, TS activities in the F-2, F-5, F-7 and F-8-treated mice were markedly suppressed to approximately 83% to 85% of that in the control ($p<0.01$ by Student's t-test) (Table 3).

Table 3. Effects of polysaccharides on TS and TK activities in tumors when orally administrated or injected i.p. for 10 days.

Polysaccharides	Enzyme activity (fmol/mg protein/min, mean ± SEM)			
	Oral administration		Injection by i.p.[a]	
	TS	TK	TS	TK
Control	321.4 ± 34.1	5.4 ± 1.3	313.3 ± 26.4	5.2 ± 1.1
F-1	305.5 ± 36.5	5.2 ± 1.4	267.5 ± 24.3	4.2 ± 0.5
F-2	237.1 ± 24.4**	3.6 ± 0.8*	178.1 ± 21.3**	2.3 ± 0.4*
F-3	305.3 ± 30.3	5.3 ± 1.4	267.3 ± 35.3	4.2 ± 0.3
F-4	318.3 ± 32.4	5.2 ± 1.2	266.7 ± 23.3	4.5 ± 0.5
F-5	287.4 ± 23.4	4.4 ± 1.1	198.6 ± 21.6**	2.6 ± 0.4*
F-6	277.8 ± 28.5	4.8 ± 1.6	264.3 ± 25.3	4.3 ± 0.5
F-7	256.3 ± 30.4**	4.6 ± 1.8	134.6 ± 12.6**	1.9 ± 03*
F-8	276.7 ± 22.1	4.5 ± 1.4	153.4 ± 13.3**	2.1 ± 0.4*
F-9	268.5 ± 18.1**	4.5 ± 1.2	136.6 ± 13.1**	1.4 ± 0.4*
F-11	278.6 ± 20.2	4.6 ± 1.2	145.6 ± 11.4**	1.6 ± 0.2*

** Significantly different from the control at $p<0.01$ by Student's t-test)
* Significantly different from the control at $p<0.05$ by Student's t-test)
[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. Samples were treated for 10 days.

Furthermore, there was big difference in TK activities with decreases in F-2, F-5, F-7 and F-8-treated mice ($p<0.05$ by Student's t-test). In cases of F-9 and F-11, the absolutely same results were observed in TS and TK activities. These results clearly indicated that the i.p. injection is much effective to suppress tumor growth than oral administration.

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