Glycoconjugate Journal 16, 247–252 (1999)

Immune-stimulating properties of polysaccharides from *Phellodendri cortex* (Hwangbek)

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Heteropolysaccarides were isolated from the Korean medicinal plant, *Phellodendri cortex* (Hwangbek), by hot water and alkali extractions. The extracted polysaccharides were fractionated into eight fractions and they are mainly composed of D-*N*-acetylglucosamine, D-galactose, D-mannose, and D-glucose. Among the polysaccharide fractions, Fr.-2 showed a potent B-lymphocyte-stimulating activity in a system using polyclonal antibody forming cells in C57BL/6XC3H mice at dosages of 2–10 mg. On the basis of their solubility in aqueous ethanol, four fractions of Fr.-2-1 to Fr.-2-4 were further obtained from the Fr.-2, and Fr.-2-3 was divided into Fr.-2-3-1, 2, 3, and 4 by DEAE cellulose chromatography. The main activity was found in Fr.-2-3-2, which contained 100% (w/w) of carbohydrates and further purified to Fr.-2-3-2-2 by gel filtration chromatography using TSK Gel HW50S. Fr.-2-3-2-2, having a molecular weight of about 230 kDa, showed the highest B-cell-stimulating activity and the half-maximal concentration for B-lymphocyte-stimulating activity was *ca.* 2.2 μg/ml.

Keywords: **polysaccharide, immune-stimulating activity, *Phellodendri cortex* (Hwangbek)**

Abbreviations: **LPS, lipopolysaccharide; DMSO, dimethyl sulfoxide; GLC, gas-liquid chromatography; PBS, phosphate buffer in saline.**

Introduction

It has been reported that the glucan-type polysaccharides, including β-glucan [1–5] and chitin [6], have antitumor activity *via* macrophage activation in the host. For example, some polysaccharides isolated from bamboo leaves, bagasse, were remarkably effective in inhibiting the growth and inducing regression of sarcoma-180 subcutaneously transplanted in mice [7,8]. This tumor-inhibiting effect was considered to be indirect and host-mediated, and not due to their cytocidal action on tumor cells. Many polysaccharides extracted from Basidiomycetes [9–14] and marine algae [15–17] have also been shown to induce antitumorial and immune-stimulating activities, thus, acting as biological response modifiers (BRM).

The medicinal plant, *Phellodendri cortex* (Hwangbek), widely found in Korea and China, has long been used for the treatment of gynecological inflammation and cancer as a traditional herb medicine having antiinflammatory, immunostimulatory and antitumor activities in oriental clinical fields in Korea. In this study, it was confirmed that the hot-water extract of the *Phellodendri cortex* (Hwangbek) showed stimulating activity against B-lymphocytes. These facts led us to characterize the active principle from the water extracts guided by an antibody forming cell (AFC) assay system.

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Materials and methods

Materials

Female (C57BL/6XC3H) F1 (B6C3F1) mice obtained from Experimental Animal Center, Korea Research Insitute of Bioscience and Biotechnology, KIST (Taejon, Korea) were maintained in the Department of Biochemistry and Molecular Biology, College of Oriental Medicine, Dongguk University, and 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 obrained from Korea Media Co., Ltd. (Seoul, Korea). Guinea pig complement and RPMI 1640 were purchased from Gibco BRL (Grand Island, NY, USA). Lipopolysaccharide (LPS) and Proteinase K (from *Tritirachium album*) were purchased from Sigma.

General analytical methods

Total sugar content was measured by the Phenol-sulfuric acid method [18]. Reducing sugars were measured by the Nelson-Somogyi method [19] using glucose (Glc) as a standard. Protein was determined by the procedure of Lowry *et al.* [20] using bovine serum albumin (BSA) as a standard. Hydrolysis of the polysaccharide was usually done by heating with 2 M sulfuric acid at 100°C 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.

Partial hydrolysis of the polysaccharide was done with 25 mM sulfuric acid (10 ml), at 100°C, 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 flame-ionization detector. Sugars were separated on a column (0.4 × 200 cm) of 3% ECNSS-M on Gas Chrom Q, at 190°C.

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 ($5 \times 10^3 - 2 \times 10^5$, Hayashibara Biochemicals Lab. Inc., Okayama, Japan) and dextrans ($2 \times 10^4 - 2 \times 10^6$, Sigma Co.,) as authentic standards of molecular weights.

Extraction, fractionation, and purification of polysaccharides

All polysaccharide fractions were prepared by repeated ethanol precipitation and dialysis against water followed by lyophilization. The dried samples were homogenized using a mechanical disintegrator with Tekmar tissue homogenizer (Tekmar Co., Cincinati, OH, USA) in 50 mM sodium phosphate buffer in saline (PBS) (pH 7.2).

The crude fraction was collected by centrifugation (15,000 xg, for 20 min) at 4°C, and delipidated with mixtures of chloroform and methanol (2:1) and (1:1), succesively (delipidated fraction, yield 72 g). The delipidated fraction (72 g) was digested with proteinase K in 50 mM phosphate buffer (pH 7.2) containing 15 mM $CaCl_2$, at 37°C, for 48 h. The digestion mixture was centrifuged and the supernatant solution was concentrated to about 120 ml, and added to ethanol (3 volumes) resulting in precipitation of a polysaccharide (Fr.-1, yield 15.2 g). The residue from the centrifugation was extracted three times with hot water at 120°C for 20 min. The extracts were combined, concentrated, and poured into ethanol (4 volumes) to give a hot water-extractable polysaccharide (Fr.-2, yield 16.8 g). The residue after extraction was treated three times with 1 M NaOH at 20°C 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 (Fr.-3, yield 4.1 g). The soluble fraction was precipitated by the addition of ethanol (Fr.-4, yield 2.4 g). The residue after extraction was treated three times with 1 M NaOH at 60°C for 6 h, in a similar fashion. From the hot alkali extract, two polysaccharide fractions (Fr.-5 1.4 g; Fr.-6, 0.6 g) were prepared in the same manner as that for Fr.-3 and Fr.-4, respectively. The alkali insoluble residue was extracted with dimethylsulfoxide (DMSO) at 60°C overnight. The extracts were dialyzed against water. The retenate was precipitated by the addition of ethanol (Fr.-7, 2.6 g), and the residue after extraction was washed thoroughly with water (Fr,-8, 10.1 g). The fractionation and extraction of the active polysaccharides are summarized in Figure 1.

Further fractionation of hot water extract (Fr.-2)

The extract (16.8 g, Fr.-2) was suspended in 500 ml of distilled water and 4 volumes of ethanol were added. This solution was allowed to settle for 2 days at 4°C. The clear yellowish supernatant was decanted off from the dark gray residue and the precipitate was concentrated for 1 h at 4°C and 5000 × g to remove remaining soluble materials. The precipitated pellet was dissolved in water and 80% ethanolic precipitation was repeated three times in the same manner. The supernatants were combined and lyophilized (11.5 g, Fr.-2-1). The final pellet was dissolved in 100 ml deionized water and dialyzed at 4°C against water for 2 days with 6 time exchanges of water. The insoluble material which

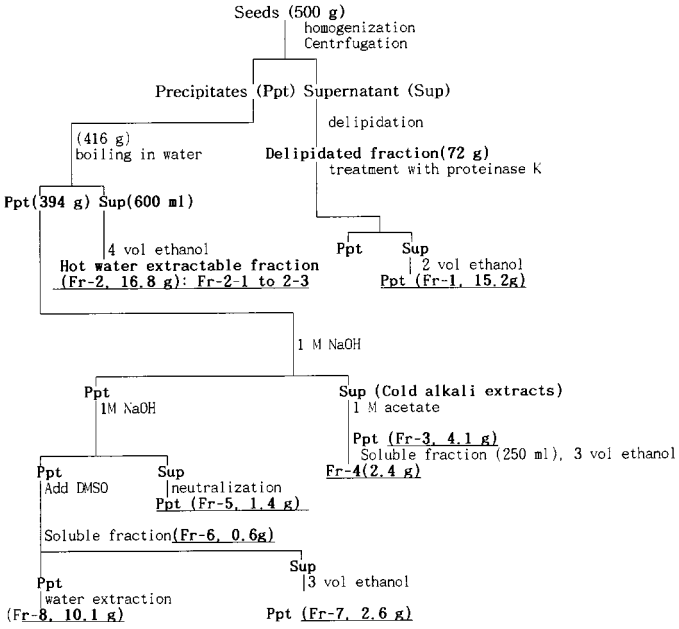

Figure 1. Fractionation of *Phellodendri cortex* (Hwangbek)

formed inside the dialysis bag was filtered off through a Whatman GF/D filter and the filtrate was lyophilized (4.8 g, Fr.-2-2). Fr.-2-2 (4.8 g) was dissolved in 50 1 of water and 150 ml of ethanol was added to make a 60% ethanolic solution. The solution was centrifuged for 30 min at 4°C and 10000 × g. After removal of ethanol, the supernatant (Fr.-2-3, 2.1 g) and pellet (Fr.-2-4, 2.3 g) were lyophilized.

a) DEAE-cellulose chromatography

One gram of Fr.-2-3 was applied to a DEAE-cellulose column (Merck Art. 3201, 3.6 × 4.3 cm) which had been equilibrated with 5 mM sodium phosphate buffer (pH 7.5). The column was initially washed with the equilibration buffer (total *ca.* 200 ml) and every 5 ml fractions was collected. The non-binding fraction which was positive to both phenol-sulfuric acid method was combined and dialized (Fr.-2-3-1, 320 mg). The bound material was eluted with a linear gradient from 0.1 to 1.0 M NaCl in the same buffer (total *ca.* 200 ml). It was finally washed with 200 ml of 1.5 M NaCl in the same buffer. The eluates were combined approximately and fractionated into Fr.-2-3-2 to Fr.-2-3-4 (52, 260 and 220 mg, respectively), as shown in Figure 2.

b) Gel filtration chromatography on TSK gel HW50S

Fraction-2-3-2 (52 mg) was dissolved in 10 ml of water then applied to a gel filtration column (1.5 × 56 cm) of TSK gel HW50S (Tosoh, Co., Tokyo, Japan) and eluted with a distilled water. Fractions of 2 ml were collected and aliquots were employed to monitor sugar and peptide contents. The tubes were combined to give fractions 2-3-2-1 to 2-3-2-3 (35, 120 and 85 mg, respectively) according to the results of bioassay and elution pattern as shown in Figure 3.

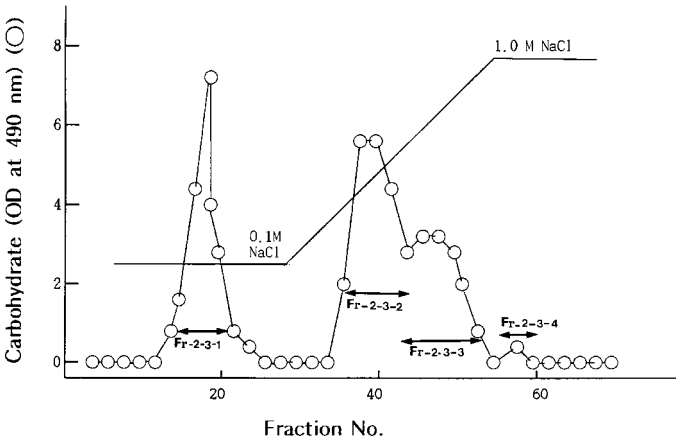

Figure 2. Chromatogram of Fr.-2-3 on a DEAE-cellulos column Each fraction was monitored by phenol-sulfuric acid method at OD 490nm (○). The gradient was started after washing with 200 ml of equilibration buffer. Fraction numbers 16–21, 37–42, 44–52 and 56–60 were collected, dialyzed and designated as Fr.-2-3-1, Fr.-2-3-2, Fr.-2-3-3 and Fr.-2-3-4, respectively. For details see Materials and Methods.

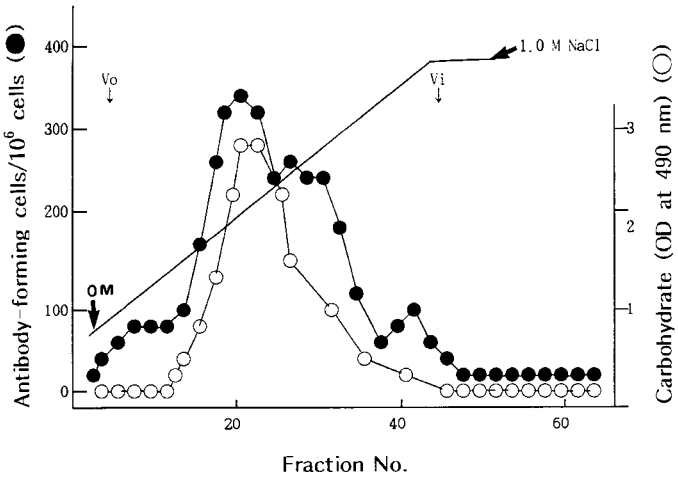

Figure 3. Elution profile of Fr.-2-3-2 on a Gel filtration column Each fraction was monitored by phenol-sulfuric acid method at OD 490nm (○). The closed circles (●) indicate the number of antibody-forming cells/10^6 cells produced by treatment with 10 μl/ml of each fraction. According to the bioassay and elution pattern, tubes 7–12, 28–22 and 23–35 were combined to give fractions Fr.-2-3-2-1, Fr.-2-3-2-2 and Fr.-2-3-2-3, respectively.

In vitro activation and AFC assay in lymphocyte

These procedures were performed as described [21]. In brief, the spleen cells were suspended in RPMI 1640 with 10% fetal calf serum and adjusted to 5 × 10^6 cells/ml. The cultures (0.5 ml aliquot/well; 4 replicate wells/treatment group) were located in individual wells of a 48-well cluster plate (Costar) and samples or LPS (25 μg/ml, purchased from Sigma) were added to them. For in vitro stimulation, the plates were incubated with rocking (8–10 rocks/min) at 37°C in Bellco (Bellco Biotech, Vineland, NJ, USA) stainless-steel tissue culture boxes in an atmosphere of 10% CO_2, 7% O_2 and 83% N_2 at 4–5 psi. The polyclonal antibody response was measured on day two after incubation. AFCs against TNP (trinitrophenyl)-haptenated sRBC were enumerated using a modified Jerne plaque assay and the cell number was counted by using a hemacytometer [14]. The number of antibody-forming cells was considered as an index of activity. The specific activity of each fraction was calculated as follows. The amounts of samples required to give the same activity as 25 μg/ml LPS were calculated from the dose-activity curves and then each activity was normalized to that of Fr.-1 taken as 1. This can be summarized by the equation: specific activity = the amount of Fr.-1 required to produce as many AFCs as 25 μg/ml LPS (10.3 mg of Fr.-1 is needed by calculation)/the amount (mg) of samples required to produces as many AFCs as LPS. Since the activity of LPS was almost saturated at 25 μg/ml, this concentration was used for all experiments as a positive control.

Table 1. Neutral sugar composition of the polysaccharide fractions from *Phellodendri cortex* (Hwangbek) extracts

	Molar ratio[b]			
Fraction[a]	*D-N-acetylglucosamine*	*D-galactose*	*D-mannose*	*D-glucose*
Fr.-1	4.1	1.0	2.6	0.6
Fr.-2	1.4	1.0	0.9	5.6
Fr.-3	0.7	1.0	5.1	4.2
Fr.-4	2.4	1.0	4.4	1.1
Fr.-5	3.7	1.0	3.2	2.0
Fr.-6	8.7	1.0	4.2	0.3
Fr.-7	2.1	1.0	6.4	1.7
Fr.-8	0.5	1.0	1.9	8.3

[a]See Materials and methods
[b]Measured by as their alditol acetates

Results and Discussion

Fractionation and chemical consitution of the extract polysaccharides of Phellodendri cortex (Hwangbek)

The extracted polysaccharides of *Phellodendri cortex* (Hwangbek) were fractionated into eight fractions, as described in Materials and Methods. Table 1 shows their neutral carbohydrate compositions, as analyzed by GLC. It was apparent that Fraction-1 (Fr.-1) solubilized from the extracts by treatment of Proteinase K constituents of D-N-acetylglucosamine and D-galactose and D-mannose in a higher proportion than that of D-glucose. The test treated with Fehling reagent showed that is did not give an insoluble copper-hydroxide complex. This shows that the mannose-rich fraction does not contain mannan as a homopolysaccharide.

B-lymphocyte-stimulating activities of the Phellodendri cortex (Hwangbek) extracts

When 0.1 to 2.0 mg/ml of each fraction were bioassayed to compare their immuno-stimulating activities in a system using polyclonal antibody forming cells in C57BL/6XC3H mice, the relative activity of Fr.-2 (2 mg) was 46% of the positive control LPS, taken as 100%, while the others showed 5 to 22% (data not shown). Table 2 shows the results obtained in assay of B-lymphocyte-stimulating activities of all polysaccharide fractions at dosages of 2 mg. Thus, among the polysaccharide fractions, the water extracted fractions, particularly Fr.-2, showed a potent B-lymphocyte-stimulating activity and Fr.-2 was considered as the active fraction to be employed for further fractionation.

Further characterization of fraction Fr.-2 by ethanol

On the basis of their solubility in aqueous ethanol, four fractions (Fr.-2-1 to Fr.-2-4) were obtained from Fr.-2 of *Phellodendri cortex* (Hwangbek). Samples containing 0.1 to 2.0 mg/ml of each fraction were bioassayed for B-lymphocyte-stimulating activity to compare their immunostimulating activities. The relative activity of Fr.-2-3 (1 mg) was 89% of the positive control LPS, taken as 100%, while the others showed values of 18 to 56% (Table 3), thus Fr.-2-3 was considered as the active fraction to be employed for further purification.

DEAE-cellulose chromatography of fraction Fr.-2-3

Further fractionation of the Fr.-2-3 was attempted by DEAE cellulose chromatography and the result afforded a non-binding fraction (Fr.-2-3-1, *ca.* 25% of Fr.-2-3 by weight) and three NaCl-eluted fractions (Fr.-2-3-2 to Fr.-2-3-4) (Fig. 2). The B-lymphocyte stimulating activity of each DEAE-cellulose fraction (0.1 to 2.0 mg/ml) was measured. The main activity was found in Fr.-2-3-2, which contained 100% (w/w) of carbohydrates (Table 4).

Table 2. B-lymphocyte stimulating specific activity of fractions from *Phellodendri cortex* (Hwangbek) extracts

Fraction	*Dose (mg)*	*Specific activity*[a]
Control	—	*1*
Fr.-1	2.0	2
Fr.-2	2.0	23
Fr.-3	2.0	12
Fr.-4	2.0	6
Fr.-5	2.0	8
Fr.-6	2.0	10
Fr.-7	2.0	12
Fr.-8	2.0	15

[a]For the evaluation of specific activity, see Materials and Methods.

Table 3. B-lymphocyte stimulating specific activity of fractions after subfractionation of Fr.-2 by ethanol

Fraction	*Dose (mg)*	*Relative activity*[a]
LPS[b]	2.0	100
Fr.-2-1	2.0	33
Fr.-2-2	2.0	18
Fr.-2-3	2.0	89
Fr.-2-4	2.0	56

[a]The positive control LPS was taken as 100%
[b]Positive control

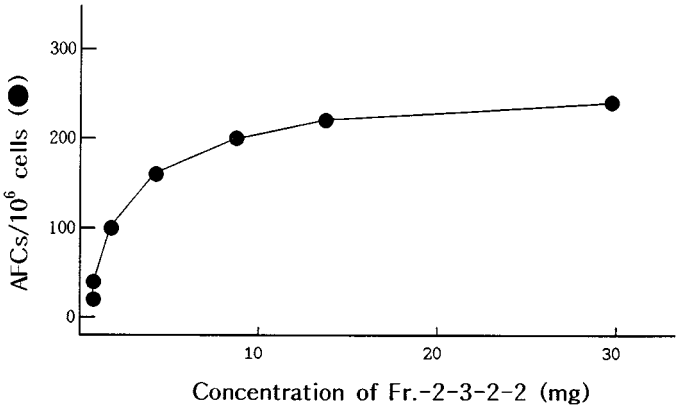

Figure 4. Dose-dependent B-lymphocyte-stimulating activity of Fr.-2-3-2-2

Gel permeation chromatography of fraction Fr.-2-3-2

Further purification of Fr.-2-3-2 was attempted by gel filtration chromatography using TSK Gel HW50S. Fifty tubes were collected and 10 μg/ml of each was applied to bioassay. On the basis of this result and elution pattern (Fig. 3), the eluates were separated into 3 fractions of Fr.-2-3-2-1, Fr.-2-3-2-2 and Fr.-2-3-2-3. Among them, Fr.-2-3-2-2, having a molecular weight of about 230 kDa, showed the highest B-cell-stimulating activity (Table 4). The half-maximal concentration of Fr.-2-3-2-2 for B-lymphocyte-stimulating activity was *ca.* 2.2 μg/ml (Fig. 4). After hydrolysis of Fr.-2-3-2-2, liberated monosaccharides were derivatized to their corresponding alditol acetates. The presence of mannose, N-acetylglucosamine, galactose and glucose were confirmed by GC as neutral sugars. Glucose, mannose and *N*-acetylglucosamine were predominant (data not shown). From these results, B-lymphocyte stimulating fraction was characterized as heteroglycan.

Recently, Song *et al.* [13] reported that the acidic heteroglycan-peptide complex produced by Basidiomycetes has an immuno-stimulating activity as a rare case. Serveral pure polysaccharides, possessing potent antitumor activity, were also reported. For example, Hirase *et al.* reported that the β-D-glucan polysaccharide of *Coriolus versicolor* is active for the anti-tumor therapy [22]. Sasaki and Takasuka confirmed the antitumor active polysaccharide as β-1,6-D-glucosyl branched β-1,3-D-glucan [23]. Many polysaccharides from Basidiomycetes have been found to be active against cancer and most of them are β-D-glucans. In plants, acidic polysaccharides of *ca.* 25 kDa containing mainly glucose and small amounts of uronic acid have been purified from leaves of *Artemisia* species [24]. These active fractions were found to activate the growth of mouse splenocytes *in vitro.* Our results indicated that the extracted polysaccharide of *Phellodendri cortex* (Hwangbek) showed the similar properties to those of above polysaccharides in B-lymphocyte stimulating activity.

Table 4. B-lymphocyte stimulating specific activity of fractions after subfractionation of Fr.-2-3 and Fr.-2-3-2 by DEAE-cellulose and Gel permeation chromatographies.

Fraction	*Dose (mg)*	*Relative activity*[a]	*Carbohydrate*[b]	*Protein*[b]	*Uronic acid*[b]
LPS[c]	2.0	100	—	0	0
Fr.-2-3-1	2.0	21	1000	0	0
Fr.-2-3-2	2.0	92	1000	0	0
Fr.-2-3-3	2.0	56	1000	0	0
Fr.-2-3-4	2.0	67	1000	0	0
Fr.-2-3-2-1	2.0	66	1000	0	0
Fr.-2-3-2-2	2.0	97	1000	0	0
Fr.-2-3-2-3	2.0	86	1000	0	0

[a]The positive control LPS was taken as 100%
[b]Presented as μg/mg and determined by the phenol-sulfuric acid, Lowry's, and the *m*-hydroxybiphenyl methods, respectively
[c]Positive control

Acknowledgment

This work was in part supported by Ministry of Health and Welfare, Korean Government.

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Received 30 December 1998, revised 17 March 1999, accepted 25 March 1999